Physiological response of *bacillus licheniformis* NCIMB 8874 to oligosaccharide elicitors

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PHYSIOLOGICAL RESPONSE OF *BACILLUS LICHENIFORMIS* NCIMB 8874 TO OLIGOSACCHARIDE ELICITORS

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ABSTRACT

The mechanism by which certain oligosaccharides elicit overproduction of secondary metabolites in *Bacillus licheniformis* was investigated. Mannan oligosaccharides (MO) derived from locust bean gum, and oligoguluronate (OG) and oligomannuronate (OM) from alginate were used in this study.

The effect of lower (50 - 25 mg L^{-1}) and higher (500 – 1000 mg L^{-1}) concentrations of MO, OG and OM on bacitracin A production was investigated. HPLC (high performance liquid chromatography) results demonstrated that the addition of lower and higher concentrations of these elicitors to cultures of *B. licheniformis* did not have an elicitation effect on bacitracin A production, compared to the optimal reported elicitor concentration (100 – 300 mg L^{-1}).

HPAEC (high performance anion exchange chromatography) and TLC (thin layer chromatography) results revealed that mannobiose, mannose and mannohexaose are the main components of MO. The addition of these saccharides, separately, to *B. licheniformis* cultures, showed that mannobiose produced the highest and sustained increase in bacitracin A concentration whereas in mannose and mannohexaose treated cultures the effects were minimal. Similar levels of bacitracin A were obtained in MO (825 mg L^{-1}) and mannobiose (800 mg L^{-1}) treated cultures. Investigation of the fate of MO during the *B. licheniformis* shake flask and bioreactor batch growth showed that MO is degraded to mannose by the enzyme ß-mannanase.

MO (300 mg L^{-1}) supplemented chemostat cultures of *B. licheniformis* did not result in a significant change in bacitracin A concentration and ß-mannanase activity compared to the control fermentation. However, when the chemostat culture was changed to batch, increases were observed both in bacitracin A concentration and ß-mannanase activity.

Incubation of *B. licheniformis* cells with oligosaccharide elicitors in microwell plates resulted in changes in Ca^{2+} influx. The highest influx was 9.2 % greater in samples incubated with MO and OG compared to the control. Addition of ionomycin (positive control) and verapamil (Ca^{+2} channel blocker) to the cultures of *B. licheniformis* resulted in 15 % increase and 74 % decrease in bacitracin A levels, respectively, as compared to the control culture.

Changes were profiled in protein expression in *B. licheniformis* supplemented with OG and MO elicitors in short and long term elicitation, using 2-Dimentional gel electrophoresis. Differentially expressed proteins were identified by LC-MS/MS. The proteins affected by short-term and long-term elicitation were mainly involved in energy generation, amino acid metabolism and stress responses. The addition of OG and MO to the cultures also altered the phosphorylation state of twelve proteins involved in energy generation, stress response and amino acid metabolism. Biochemical assays revealed changes in reactive oxygen species levels as well as antioxidant enzymes, superoxide dismutase and catalase in *B. licheniformis* cultures supplemented with elicitors.

The novel findings of this work have advanced understanding of the mechanism of action of elicitors in *B. licheniformis* cultures.

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To my Holy Father God
Beloved Family &
Precious Husband

Entrega o teu caminho ao Senhor;
confia nele, e ele tudo fará (Sl 37:5)

AUTHORS DECLARATION

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another university or similar institution.

Any views expressed in this work are those of the author and in no way represent those of the University of Westminster.

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2-D	Two-dimensional
2-DE	Two-dimensional electrophoresis
4`PAN	Phosphopantethene arm
µ	Specific growth rate
Ahp	Alkyl hydroperoxide reductase
ATP	Adenosine triphosphate
AM	Acetoxymethyl
ANOVA	Analysis of variance
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CAM	Calmodulin
CAMP	Cyclic adenosine monophosphate
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1- propanesulfonate
CO_2	Carbon dioxide
cyclic GMP	Nucleotide of guanosine
DA	Decursinol angelate
DCFH-DA	2'7'-Dichlorodihydroflurescein diacetate
DG	2.4-diacetylphloroglucionol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DnaK	Chaperone protein DnaK
DON	Deoxynivalenol
DOT	Dissolved oxygen tension
DP	Degree of polymerisation
DTT	Dithiothreitol
EF-Tu	Elongation factor thermo unstable
EF-Ts	Elongation factor thermo stable
EF-G	Elongation factor G
EPS	Extracellular polysaccharide
GA	Ganoderic acid
GOD	Glucose oxidase
GSH	Gluthathione
H^+	Hydrogen ions
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPAEC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IPG	Immobilised pH gradient
IP_3	Inositol trisphosphate
IPN	Isopenicillin
KDa	Kilo Dalton
L	Litre
LB	Luria Bertani
LBG	Locust bean gum
LC-MS/MS	Liquid chromatography–mass spectrometry
LLD-ACV	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine
M	Molar
MAPK	Mitogen activated protein kinase
MO	Mannan oligosaccharide
MS/MS	Tandem mass spectrometry
MW	Molecular Weight
NAD^+	Nicotinamide adenine dinucleotide coenzyme

NADH	Nicotonamide adenine dinucleotide
NCIMB	Natural Collection of industrial and Marine Bacteria
NCBI	National Centre of Biotechnology Information
NCBInr	National Centre of Biotechnology Information database
NDK	Nucleoside diphosphate kinase
nm	Nanometer
O_2	Oxygen
O^-_2	Superoxide anion
OD	Optical density
OG	Oligoguluronate
OM	Oligomannuronate
pI	Isoelectric point
PTM	Post translational modification
PTS	Phosphoenolpyruvate: carbohydrate phosphotransferase transport system
PKC	Protein kinase
PSAT	Phosphoserine aminotransferase
RFU	Relative fluorescence units
RLU	Relative luminescence units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF	Shaken flasks
SOD	Superoxide dismutase
STR	Stirred tank reactor
TCA	Citric acid cycle
T_d	Doubling time
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	Thin layer chromatography
U	Unit
UV	Ultraviolet
UVA	Ultraviolet A
VOCCs	Voltage operated calcium channels
vvm	Volume per volume per minute
v/v	Volume per volume
w/v	Weight per volume

AIM

The aim of this work was to explore the physiological response of *Bacillus licheniformis* NCIMB 8874 to oligosaccharide elicitors.

To address the aim, the following studies were carried out:

1. To evaluate the effect of lower and higher concentrations of oligosaccharide elicitors on growth of *B. licheniformis* and bacitracin A production.

2. To investigate the fate of MO elicitor in the liquid culture of *B. licheniformis*.

3. To investigate the potential role of the degradation products of MO (mannose, mannobiose and mannohexaose) as elicitors in liquid cultures of *B. licheniformis*.

4. To evaluate the effect of MO on *B. licheniformis* under continuous culture.

5. To investigate the elicitation effects on intracellular calcium levels in *B. licheniformis* cultures.

6. To assess the effects of elicitor(s) on the expression and phosphorylation level of cytoplasmic proteins of *B. licheniformis*.

Chapter I

1.0 Introduction

1.1 Biotic and abiotic elicitation: Potential for industrial applications

Despite the fact that microbes are extremely good sources of a wide range of bio compounds, these compounds are usually produced in small amounts for their own benefit. Bio-industries are interested in the enhanced production of these small-volume high-value compounds produced by a number of diverse organisms. Research has been mainly focused on strain selection and improvement, media development, metabolic engineering through the application of recombinant DNA technology, pathway engineering, and bioreactor design. Nevertheless, another promising technique, not explored extensively, is based on the application of biotic and abiotic elicitation.

Elicitation is a method where the addition of stress-inducing agents (elicitors) enhances primary and secondary metabolite production in plants and microorganism. Elicitors can be classified into two main categories - abiotic (physical or chemical) and biotic (chemical with complex or defined composition). By definition, abiotic elicitors are inert factors that may or not damage the living organisms. Examples of these non-living factors include, oxidative, heat, osmotic and acid stress (Thammavongs *et al*., 2008) whereas biotic elicitors are originated from living factors, such as elicitors derived from protein, lipids and oligosaccharides. Examples include elicitins, lipopolysaccharides and mannan oligosaccharides (Radman *et al*., 2003) (Table 1.1).

Table 1.1 Elicitors of plants and microbial cells (Modified from Radman *et al*., 2003).

<table>
<tr><th colspan="6">Elicitors</th></tr>
<tr><td rowspan="2">Abiotic</td><td>Physical elicitors</td><td colspan="4">UV rays, oxidative, pH, heat, osmotic and starvation stress</td></tr>
<tr><td>Chemical elicitor</td><td colspan="4">Metal ions (lanthanum, europium, calcium, silver, cadmium), oxalate</td></tr>
<tr><td rowspan="14">Biotic</td><td rowspan="14">Chemical elicitor</td><td>Complex composition</td><td colspan="3">Yeast cell wall, mycelial cell wall, fungal spores</td></tr>
<tr><td rowspan="13">Defined composition</td><td rowspan="9">Carbohydrates</td><td rowspan="5">Polysaccharides</td><td>Alginate</td></tr>
<tr><td>LBG</td></tr>
<tr><td>Pectin</td></tr>
<tr><td>Chitosan</td></tr>
<tr><td>Guar gum</td></tr>
<tr><td rowspan="4">Oligosaccharides</td><td>Mannuronate</td></tr>
<tr><td>Guluronate</td></tr>
<tr><td>Mannan</td></tr>
<tr><td>Galacturonides</td></tr>
<tr><td rowspan="3">Proteins</td><td>Peptides</td><td>Glutathione</td></tr>
<tr><td rowspan="3">Proteins</td><td>Cellulase</td></tr>
<tr><td>Elicitins</td></tr>
<tr><td>Oligandrin</td></tr>
<tr><td>Lipids</td><td></td><td>Lipopolysaccharides</td></tr>
<tr><td>Glycoproteins</td><td></td><td>Not characterised</td></tr>
<tr><td>Volatiles</td><td></td><td>C6-C10</td></tr>
</table>

To date, a significant number of microorganisms have been reported in the literature as being able to accumulate enhanced amounts of fermentation products after altering their metabolic temperature, cellular redox state, nutritional conditions (abiotic elicitation), and through the addition of small amounts of biotic oligosaccharides based elicitors. The production of a broad range of bio products with economic importance such as antibiotics, enzymes, pigments and mycotoxins have been positively affected by elicitors (Omori *et al*., 1996; Nakata *et al*., 1999; Radman *et al*., 2003; Murphy *et al*., 2007a; Sumathy *et al*., 2007; Liang *et al*., 2009a,b).

Successful application of cost effective abiotic and biotic elicitors would have notable economic benefits for the biotechnology and pharmaceutical industries

where production of commercially essential bio products demands lower-cost and higher-yield processes. Whilst successful approaches have been used for industrial improvement of microbial metabolites, a cheap alternative is the application of abiotic and biotic elicitors. In order to survive under stress conditions, microorganisms have, as instantaneous adaptive responses, developed morphological and physiological modifications. Stress response is a complex phenomenon that differs depending on the type of microorganism and the type of the stress (Storz and Hengge-Aronis, 2000). Overall, microorganisms defend themselves against unfavourable conditions by inducing the expression of stress related proteins that protect the cells against adverse effects. While a range of biotic elicitors and their effects have been extensively reported (Ariyo *et al*., 1997; Petruccioli *et al*., 1999; Tamerler *et al*., 2001; Nair *et al*., 2008) the regulation of the microbial stress defence in biotic elicitation resulting in the enhancement of metabolites is not completely understood. This study investigates some of the physiological and metabolic responses caused by oligosaccharide biotic derived elicitors in cultures of *Bacillus licheniformis*.

1.2 Abiotic elicitation: Effects on the microbial metabolite production

1.2.1 Osmotic, Heat and pH stress

Abiotic factors such as osmotic, heat and pH stress have been successfully used to improve production of microbial metabolites by many researchers (Table 1.2) (Fiedurek, 1998; Nakata *et al*., 1999; Taherzadeh *et al*., 2002; Marova *et al*., 2004; Sevcikova and Kormanec, 2004; Varela *et al*., 2004; Sumathy *et al*., 2007; Liang *et al*., 2009a).

Table 1.2 Examples of Abiotic stress in microbial cultures.

Abiotic Stress	Microorganism	Metabolite affected	Reference
Oxidative	*S. salmonicolor*	Carotenoides Ergosterol	Marova *et al*., (2004)
	C. utilis	Gluthathione	Liang *et al*., (2009a)
	B.trispora	Carotene	Nanou and Roukas, (2009)
Osmotic	*A. niger*	Glucose oxidase	Fiedurek, (1998)
	P. fluorescens	Pyoluteorin 2-4 diacetylphloruglucionol	Nakata *et al*., (1999)
	S. cerevisiae	Glycerol	Carvalheiro *et al*., (1999)
	S. coelicolor	Undecylprodigiosin	Sevcikova and Kormanec, (2004)
	M.echninospora	Gentamicin	Himabindu *et al*., (2007)
	M. purpureus	Pigments	Sumathy *et al*., (2007)
	C. utilis	Glutathione	Liang *et al*., (2009b)
Heat	*S. venezuelae*	Jadomycin B	Doull *et al*., (1993)
	S. cerevisiae	Glycerol	Omori *et al*., (1996) Omori *et al*., (1997); Kajiwara *et al*., (2000)
	P.fluorescens	Pyoluteorin 2-4 diacetylphlorogeucionol	Nakata *et al*., (1999)
	M. purpureus	Pigments	Sumathy *et al*., (2007)
pH	*A. flavus*	Aflotoxin	Cotty, (1988)
	S. coelicolor	Methylenomycin	Hayes *et al*., (1997)
	S. kasuggaensis	Kasugamycin	Kim *et al*., (2007)
	S. hygroscopicus	Geldanamycin	Song *et al*., (2008)
	M. purpureus	Red pigments	Orosco and Kilikian, (2008)
	F. graminearum	Deoxynivalenol	Gardiner *et al*., (2009)
	A. nidula	Sterigmatocystin	Delgado-Virgen and Guzman-de-Peña, (2009)
Starvation	*D. hansenii*	Xylitol	Tavares *et al*., (1999)
	A. gossypii	Riboflavin	Schlosser *et al*., (2007)
	T. versicolor	Lacasse	Hailei *et al*., (2009)

These studies cover a variety of strains and microbial metabolites. Fiedurek, (1998) investigated the effect of osmotic stress on glucose oxidase (GOD) production by *Aspergillus niger*. The author demonstrated that the addition of NaCl to the culture improved the production of GOD enzyme by 2.1 fold in comparison to the control culture. Gluthathione production was also increased by 71 % in NaCl supplemented cultures of *Candida utilis* (Liang *et al*., 2009b). The production of gentamicin, an important antibiotic used to treat many types of bacterial infections, was enhanced by 1.26 fold in cultures of *Micromonospora echinospora* stressed with NaCl (Himabindu *et al*., 2007). In addition, Asthana *et al*., (2005) suggested that NaCl was able to trigger trehalose production in cultures of *Cyanobacterium anabaena 7120*.

The effect of abiotic elicitor on the pigment production of *Monascus purpureus* was also investigated. Red pigment production was improved sixteen times when pH stress was applied to the fungal culture *(*Orosco and Kilikian, 2008). A similar result was obtained in solid state, with accumulation of pigments when *M. purpureus* was exposed to osmotic stress (Sumathy *et al*., 2007).

Osmotic and heat shock treatment were also demonstrated to have positive effects on the pyoluteorin (PT) and 2.4-diacetylphloroglucionol (DG) antibiotic production in cultures of *Pseudomonas fluorescens S272*. Nakata *et al*., (1999), reported an improvement of two fold increase in the antibiotic production when NaCl was added to the microbial culture. They also described an even higher concentration of both metabolites (two to three fold increase) when the cells were exposed to the heat shock treatment.

The exploitation of stress factors for the enhancement of metabolite synthesis by *Streptomyces spp*. have been extensively studied (Doull *et al*., 1993; Hayes *et al*., 1997; Kim *et al*., 2000; Sevcikova and Kormanec, 2004). Hayes *et al*., (1997) reported that methylenomycin production by *S. coelicolor* was promoted by acidic pH shock whereas in *S. kasugaensis* cultures a seven fold increase was observed in the kasugamycin production by pH stress (Kim *et al*., 2000). Doull *et al*., (1993) described similar results where the biosynthesis of jadomycin B was enhanced by ethanol treatment and heat shock in cultures of *S. venezuelae*.

Geldamycin production was also improved by the application of pH shock in cultures of *Streptomyces hygroscopicus subsp. duamyceticus* (Song *et al*., 2008).

Production of the pigmented antibiotics actinorhodin and undecylprodigiosin was also reported under NaCl stressed conditions in cultures of *S. coelicolor A3*. In this case, the NaCl differentially affected the production of the two products. Sevcikova and Kormanec (2004) demonstrated that at high salt concentration, actinorhodin production was inhibited and undecylprodigiosin was improved, reaching a maximum production of 3.5 µg ml^{-1} against 0.5 µg ml^{-1} in the control cultures.

Glycerol, another metabolite widely used in the biotechnology industry (Taherzadeh *et al*., 2002) was reported to be affected by abiotic elicitors. Omori *et al*., (1996) and Omori *et al*., (1997) stated a maximum improvement in glycerol production of around 1.25 fold over the control cells, as a result of heat shock treatment of the culture of *Saccharomyces cerevisiae*. Carvalheiro *et al*., (1999), showed similar results. A greater improvement of glycerol production (20 %) was obtained by Kajiwara *et al*., (2000) under similar conditions. In addition, osmotic changes were reported to promote glycerol accumulation in *Debaryomyces hansenii* cultures (Andre *et al*., 1988).

Mycotoxins are toxic secondary metabolites produced by groups of filamentous fungi (i.e., *Aspergillus spp.* and *Fusarium spp.*) (Schmid-Heyd *et al*., 2008) which are responsible for a loss in agricultural production of around $ 1.5 billion every year (Yole and Williams, 2008). Cotty, (1988); Delgado-Virgen and Guzman-de-Peña, (2009); Gardiner *et al*., (2009) demonstrated that pH stress can have a profound effect on the biosynthesis of mycotoxins. Cotty, (1988) suggested that pH stress mediated the regulation of aflotoxin biosynthesis. Aflotoxin is a mycotoxin which frequently contaminates agricultural field. The authors described an improved production of around tenfold as compared to the control, when pH stress was applied in cultures of *Aspergillus flavus*. Gardiner *et al*., (2009) reported comparable results in the production of another harmful mycotoxin deoxynivalenol (DON), when low extracellular pH was employed in the growth to *Fusarium graminearum*. Delgado-Virgen and Guzman-de-Peña

(2009), showed similar results. The authors demonstrated that pH stress could positively affect the sterigmatocystin production the cultures of *Aspergillus nidulans*.

1.2.2 Starvation stress

Starvation stress has been described as an alternative method for the improvement of microbial metabolites. Tavares *et al*., (1999) reported an increase in xylitol production when phosphate limitation was applied to the cultures of *Debaryomyces hansenii*. Similar results were described for production of riboflavin. In addition, the production of an important industrial enzyme was similarly affected by starvation conditions. Lacasse synthesis was enhanced 11.8 times in cultures of *Trametes versicolor* as a result of glucose starvation (Hailei *et al*., 2009).

1.2.3 Oxidative stress

Studies involving oxidative stress aiming at overproduction of microbial metabolites have been reported, with few but significant examples (Marova *et al*., 2004; Liang *et al*., 2009a).

Carotenoids are important colorants used by food industry. They are mainly produced through chemical synthesis. However, in the last decade biosynthesis of carotenoids through large-scale cultures of plants or microbes was considered (Sandmann, 2001; Das *et al*., 2007; Delcampo *et al*., 2007). Marova *et al*., (2004) reported an improvement of carotenoids and ergosterol pigments production by *Saccharomyces salmonicor*, when the oxidative agent (H_2O_2) was added to the culture. They described a production of 1.44 $mg.g^{-1}$ of ergosterol in test culture against 0.31 $mg.g^{-1}$ of the control.

Another example is the application of oxidative stress for overproduction of gluthathione (GSH); an important antioxidant that helps protect cells from reactive oxygen species (ROS). Liang *et al*., (2009a) described an improvement of the GSH production in yeast strain *Candida utilis*. They demonstrated that H_2O_2 stimulates GSH accumulation and maximises GSH yield by 58 % higher than the control in shaken flasks cultures.

1.3 Biotic Elicitation: Effect on the microbial metabolite production

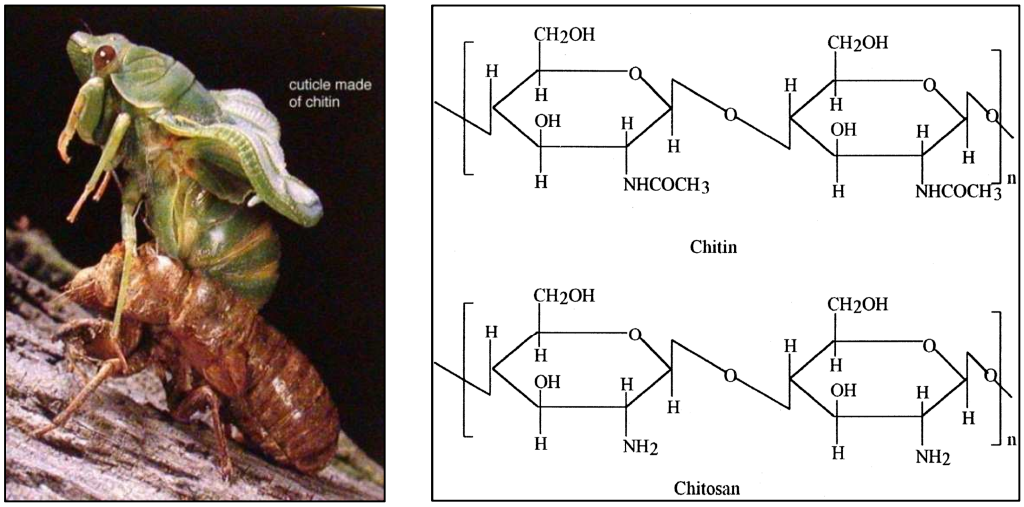
1.3.1 Carbohydrates as elicitors

Carbohydrates can be separated into four main groups: monosaccharide, disaccharides, oligosaccharides and polysaccharides. Monosaccharides (most basic units) act as the building blocks for the other groups. Disaccharides are formed of two monosaccharide units, whereas polysaccharides with more complex polymeric structures are formed by a number of repeating units (either mono- or di-saccharides) linked by either α or β glycosidic bonds. Oligosaccharides are polymers of three to twenty monosaccharide units.

The degree of polymerisation (DP) refers to the number of units obtained upon hydrolysis. As maltose is composed of two molecules of glucose (monosaccharide), it is referred to as a disaccharide as it has a DP of two. In this study, oligosaccharide elicitors were prepared by enzymatic hydrolysis of galactomannans (Locust bean gum) and by acid hydrolysis of Alginate.

1.3.1.1 Chitin and Chitosan

Chitin is the main component of fungal cell walls, the exoskeletons of crustaceans, insects and molluscs (Figure 1.1). Composed of long-chain of *N*-acetylglucosamine units covalently linked by β -1, 4 linkages; chitin is known to be the second most abundant natural biopolymer found in nature (Sandhya *et al*., 2004).



http://www.photoresearchers.com http://cool.conservation-us.org

(A) **(B)**

Figure 1.1 (A) A cicada sheds its chitinous exoskeleton (B) Chitin and chitosan schematic diagram.

Chitosan is the deacetylated form of chitin and is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Sandhya *et al*., 2004). Chitin and chitosan have been used in a wide range of products, including in food and pharmaceutical industries (Shahidi *et al*., 1999). For instance, due to their high percentage of nitrogen, antimicrobial properties, and their characteristic viscosity when neutralised with acids, chitin and chitosan are mainly used as emulsifying agent in creams and lotions (Dutta *et al*., 2004). The role of chitin and chitosan as elicitors and the capability to induce secondary metabolite production in plants is well known (Amborab *et al*., 2008; Orlita *et al*., 2008; Lizárraga-Paulín *et al*., 2011).

1.3.1.2 Pectins

Pectins are a family of complex polysaccharides found in plants (between 0.5 – 4.9 % of the fresh weight) (Kashyap *et al*., 2001; Jayani *et al*., 2005). Pectin consists mainly of 1, 4-linked α-Dgalactosyluronic residues (Sharma *et al*., 2006), being galacturonan, rhamnogalacturonans and homogalacturonan, the main pectic polysaccharides isolated from primary plant cell walls (Whitaker, 1990).

The application of pectin in the food and pharmaceutical industry is vast. Due its ability to form gels in the presence of Ca^{2+} ions, in food industry, pectin is used in jams, jellies, frozen food whereas in the pharmaceutical industry, it is used to reduce blood cholesterol levels and gastrointestinal disorders (Thakur *et al*., 1997). Similarly as for chitin and chitosan, pectins are well known to act as elicitors in plants (Fernandez-Acero *et al*., 2010; Pec *et al*., 2010) and fungal cultures (Radman *et al*., 2003).

1.3.1.3 Locust bean gum and Mannan oligosaccharides

Mannan shares a significant portion of the hemicellulose fraction of plant cell walls. Hemicellulose is polysaccharides mainly composed of mannan, xylan and galactan (Dhawan and Kaur, 2007).

Galactomannans are polysaccharide polymers derived from the seed endosperm of leguminous plants such as *Ceratania siliquia* or guar *Cyamopsis tetragonolobus* (Figure 1.2). They are composed of β-(1, 4) – linked mannan chains-forming a linear chain polymer with a α-(1, 6) - galactosyl side chain residue (McCleary, 1979). Galactomannans solubility and viscosity are directly related to the mannose/galactose ratio, which can vary from 1 to 5 (Reid, 1985).

http://commons.wikemedia.org

Figure 1.2 *Ceratonia siliqua* (carob tree) that contains Galactomannans.

Locust beam gum (LGB) is galactomannan extracted from carob tree and contains one galactose per five units of mannose, making it soluble only in hot water. Due to its property, LGB is commercially available and is used as a gelling, stabilising and thickening agent. LBG has been reported to enhance secondary metabolites in *P. chrysogenum* cultures (Ariyo *et al*., 1998; Asilonu *et al*., 2000). Mannan oligosaccharides (MO) used in this study was prepared by the enzymatic hydrolysis of LBG with β-D-mannanase enzyme (Figure 1.3). This enzyme is capable of cleaving the side chain of LGB randomly producing manno or galacto-manno oligosaccharides (Dekker and Richards, 1976).

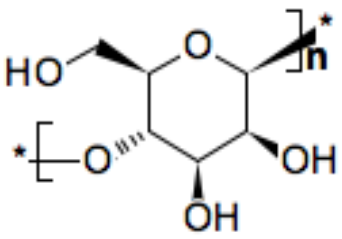


Figure 1.3 Schematic diagram of mannan oligosaccharides (MO).

Yeasts are also good sources of mannan oligosaccharides, which are one of the major components of the yeast cell wall together with beta glucans. MO has been industrially extracted from *Saccharomyces cerevisiae* and *Candida spp*. and is currently commercially available as an animal feed additive (Midlant, 2011).

MO as an additive, is reported to be capable of promoting proliferation of beneficial bacterial flora (e.g, *Bifidobacteria*) by decreasing pathogenic bacteria of the animal's gut and therefore enhancing the function of digestive system (Yalcinkaya *et al*., 2008). Mannan oligosaccharides were reported to improve growth in broiler (Hooge, 2004a), turkey (Hooge, 2004b), and swine (Miguel *et al*., 2002) by balancing the intestinal environment and by stimulating the immune response of the animals.

This is possible due to the ability of beneficial bacteria such as *Bifidobacteria* and *Lactobacilli* to degrade MO while neither the digestive system of monogastric animals (by means of acid or enzyme production) nor the pathogenic bacteria (*Clostridia* spp., *E. coli* and *Samonella* spp.) can perform this task. Most of the pathogenic bacteria possess surface adhesion or lectins (carbohydrate binding-proteins) which attach to the oligosaccharide component of glycoconjugate receptors present on the surface of epithelial cells. In the presence of MO, the attachments of bacterial pathogens to the mucosal surface is reduced since the pathogens may be bound to MO's mannose sites and therefore fail to settle on the epithelial cells (Figure 1.4) (Yalcinkaya *et al*., 2008). Therefore, yeast mannan is beneficial to animals since it prevents pathogenic bacteria from binding to the gut wall and causing diseases.

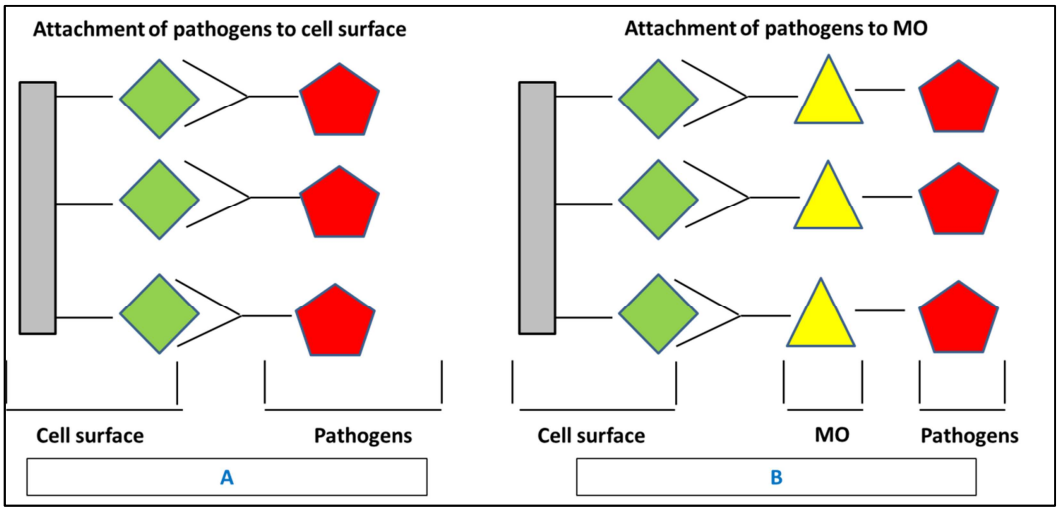


Figure 1.4 Mechanism of action of MO as an animal food additive. (A) Attachment of pathogens to cell surface. (B) Attachment of pathogens to oligosaccharides (Yalcinkaya *et al*., 2008).

1.3.1.4 Alginates and Oligoguluronate and oligomannuronate elicitors

Alginates are normally available as monovalent salts of alginic acid extracted from different types of brown seaweed such as *Laminaria hyperborea* and *Macrocystis pyrifera* (Figure 1.5) although some bacterial species such as *Azotobacter chrococcum* (Khanafari and Sepahei, 2007) and *Pseudomonas aeruginosa* (Anastassiou *et al*., 1987) are also capable of producing extracellular alginate.

Alginates are linear unbranched polymers containing β-(1, 4)-linked D-mannuronic acid and α-(1, 4)-linked L-guluronic acid residues (Nelso and Cretcher, 1929; Gacesa, 1987). These two unites are joined by glycosidic linkages at the C1-C4 position. Alginate exists as homopolymer or heteropolymer blocks. Homopolymer blocks could be either only OG (Figure 1.6) or OM (Figure 1.7). Heteropolymers are combined units of OG/OM (Haug *et al*., 1974).

A **B**

http://www.seaweed.ie http://www.seaweed.ie

Figure 1.5 *Laminaria hyperborean* (A) and *Macrocystis pyrifera* (B) containing alginate.

Alginates are used in a variety of products including those in pharmaceutical and food industry. Alginates are added as stabiliser in ice cream and juice industries; and are used extensively as a matrix for cell immobilisation (Mosahebi *et al*., 2001), as drug carriers (Tonnesen and Karlsen, 2002), wound dressings (Thomas, 2000) and in transplantation therapy (Fu *et al*., 2003). The biological activity of alginates on microbial metabolites is also known (Petruccioli *et al*., 1999). Oligoguluronate (OG) and oligomannuronate (OM) elicitors used in this study were prepared from acid hydrolysis of alginates. Figures 1.6 and 1.7 show the schematic diagrams of oligoguluronate (OG) and oligomannuronate (OM) elicitors.

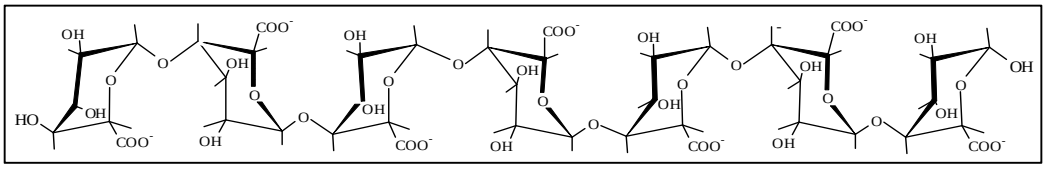


Figure 1.6 Schematic diagram of Oligoguluronate (OG).

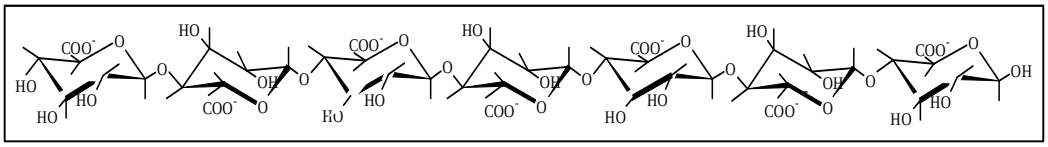


Figure 1.7 Schematic diagram of Oligomannuronate (OM).

1.3.2 Biotic elicitation in fungal systems

Earlier reports on microbial-elicitation were mostly on fungal cultures of *Penicillium chrysogenum.* In 1997, Ariyo and co-workers claimed an improvement of penicillin G production in the fungal culture as results of the addition of different degrees of polymerisation of the alginate-derived oligosaccharides. Later, the same group reported an enhancement of 47 and 49 % of the same antibiotic as a result of OG and OM addition, respectively (Ariyo *et al*., 1998). Changes in the extracellular concentrations of the secreted intermediates of penicillin biosynthesis (δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) and isopenicillin (IPN)) were also reported for the elicited cultures (Tamerler *et al*., 2001).

Due to the commercial importance of penicillin G as one of the main antibiotics in the world market (Brakhage, 1998; Penalva *et al*., 1998) *P. chrysogenum* was targeted in several studies involving oligosaccharide elicitors. Investigation using uronic acid oligosaccharides demonstrated an improvement in the production of penicillin G by around 46 % (Asilonu,1999), while locust beam gum derived elicitor resulted in 130 % increase in the antibiotic production in bioreactor studies involving *P. chrysogenum* (Tamerler *et al*., 2001).

Studies concerning mannan oligosaccharide elicitors also showed positive effect on penicillin G production (69 % increase) concomitant with no traceable changes in the cell dry weight between the control and elicited cultures (Radman *et al*., 2004a). Later investigations (Radman and Keshavarz, 2004b) showed that the addition of the oligosaccharide elicitors encouraged the onset of sporulation and spore production in this strain. Highest spore concentrations were obtained by MO followed by OM and OG supplemented cultures (Radman, 2002). Likewise a connection between penicillin G biosynthesis and morphological changes in *P. chrysogenum* was also observed. The addition of the oligosaccharide elicitor to the fungal culture increased the hyphal tip number by up to 47 % while an increase of 120 % in penicillin G levels was reached.

Since penicillin G production takes place at the region near the tips of the hyphae the authors speculated that the mechanism by which elicitors causes

morphological changes in *P. chrysogenum* may be similar to those reported when paramorphogens are added in fungi cultures (Radman, 2002).

Paramorphogens are substances capable of inducing morphological changes in fungi. Jones, (2007) showed a positive correlation between the amount of mycelium branching and accumulative levels of phenol oxidase in cultures of *Pyconoporus cinnabarinus*, both induced by the presence of the paramorphogens, Remazol Brilliant Blue R dye, lithium acetate and cellobiose. The author supports the close correlation between hypha tip density and branching which predict the accumulation levels of phenol oxidases in the fungi cultures.

Physiological examination of the effects caused by the elicitors in the cultures of *P. chrysogenum* showed that production of reactive oxygen species (ROS) was affected. In this case the addition of mannan oligosaccharides elicitor decreased the production of ROS by up to 54 %. The authors suggested that ROS might be involved in the overproduction pathway of the penicillin G by the elicitors (Radman *et al*., 2004a).

Further elicitation studies concerning *P. chrysogenum*, showed a significant increase in the transcriptional levels of the penicillin G biosynthetic genes (*pcb*AB, *pcb*C and *pen*DE) in elicitor supplemented cultures, indicating that the effects caused by the elicitor, are at transcriptional level. They observed an increase in the cytosolic calcium levels as a consequence of the elicitor's addition (Nair, 2007).

The effect of different elicitors on pigment production was also reported in strains of *P. chrysogenum* (Asilonu *et al*., 2000; Nair *et al*., 2005). The yellow pigment production (chrysogenin) was increased by up 55 % and 27 % in two different strains of *P. chrysogenum* as results the MO addition (Nair *et al*., 2005).

Further elicitation studies were conducted for production of glucose oxidase. Petrucciolo *et al*., (1999) reported an increase of 70 % on the production of GOD in cultures of *P. variabile*. GOD has been widely used in paper test strips for diabetic patients and in production of biosensors (Eun-Hyung and Soon-Youn, 2010).

Production of a valuable oriental medicine, ganoderma extract (intracellular (IPN) and extracellular (EPS) polysaccharides) and ganoderic acid (GA) used to treat many diseases such hypertension, hypercholesterolemia, leukaemia and gastric cancer were also investigated under elicitation conditions (Ghorashi, 2004). The author reported an enhancement of around 70 %, 38 % and 42 % in IPN, EPS and GA production, respectively, in cultures of *Gonoderma G991* as a result of MO supplementation.

Laccases are enzymes well known for their vital role in the textile and leather industry as well as their wide application as lignin and pollutant degrading enzymes (Raghukumar *et al*., 2008). The effect of oligosaccharide elicitors on production of lacasses by three different white-rot fungi, *Pycnoporus sanguineus*, *Coriolopsis polyzona* and *Pleurotus ostreatus*, were investigated. Enhancement of laccases in liquid cultures of *P. sanguineus,* and *P. ostreatus* were 88 fold and 2 fold higher in the MO supplemented cultures, respectively, as compared to the control cultures (Vanhulle *et al*. 2007).

1.3.3 Biotic elicitation in bacterial systems

To date, elicitation studies in bacterial systems have been mostly carried out in strains of *Streptomyces spp.* (Sangworachat, 2006) and *Bacillus licheniformis* (Murphy *et al*., 2007a,b; Murphy, 2008). The addition of MO to liquid cultures of *S. rimosus, S. coelicolor and S. avernitilis* resulted in an increase of 13 %, 119 % and 64 % on the production of the oxytetracycline, actinorhodin and avermectin antibiotics, respectively; the increase was concomitant with no changes in biomass production as compared to control culture. Significant effect on morphological pattern, transcriptional and physiological levels were also observed. A decrease in the production of reactive oxygen species with a concomitant increase in catalase activity were described (Sangworachat, 2006).

Elicitation studies concerning *B. licheniformis* cultures were also conducted. Murphy and co-workers (2007a) established that addition of OG, OM and MO enhanced the production of the antibiotic bacitracin A in the Gram-positive bacterium *B. licheniformis* by 29 %, 27 % and 16 %, respectively. Significant increase was observed in the transcriptional levels of the bacitracin biosynthetic

and ABC transporter genes, where a higher transcript levels of *bacABC* and *bcrABC* in the elicitor-added cultures compared to the control culture was found (Murphy *et al*., 2007b). Additionally, Murphy, (2008) suggested that the biotic elicitors effect a decrease in ROS levels and an increase catalase activity.

Recent work showed that the presence of the elicitors affected the cytosolic calcium levels of *Bacillus subtilis* and *Escherichia coli*. OG and MO generated an increase of 11 and 7 fold on cytosolic calcium levels in *E. coli* whereas in *B. subtilis* the increase was 10 and 3 fold, respectively (Murphy *et al*., 2011).

A summary of the responses generated by the interaction between the microbial cells and biotic elicitors (oligosaccharides elicitor) reported in literature is given in Table 1.3.

Table 1.3 Elicitation studies in filamentous microbes (Modified from Murphy, 2008).

Microorganism	Elicitor addition	Metabolite effected	Reference
P.chrysogenum	OG/OM/MO	Penicillin G	Ariyo *et al*., (1997;1998)
P. variable	MO	Glucose oxidase	Petruccioli *et al*., (1999)
P. chrysogenum	OG/OM/MO	Penicillin G	Tamerler *et al*., (2001)
P. chrysogenum	MO	Penicillin G	Radman, (2002)
Gonoderma G991	MO	IPN EPS GA	Ghorashi, (2004)
P. chrysogenum	MO	Penicillin G	Saez, (2005)
S. coelicolor *Sc. erythreae* *S. rimosus* *S. avermitilis*	MO	Actinorhodin Erythromycin Oxytetracycline Avermectin	Sangworachat, (2006)
P. sanguineus *C. polyzona* *P. ostreatus*	MO	Laccases	Vanhulle *et al*., (2007)
B. licheniformis	OG/MO	Bacitracin A	Murphy *et al*., (2007a)
P. chrysogenum	MO	Penicillin G Chrysogenin	Nair (2007)

Based on the overall findings, a hypothetical mechanism for elicitation response was proposed in fungal (Radman *et al*., 2003) and bacterial systems (Murphy, 2008). The proposed mechanism in fungi states that the elicitors mimic "hormone-cell" interaction. The presence of elicitors in fungal cultures results in the binding of the elicitor to a specific cell membrane receptor which transmits such perceived signal through alteration in the Ca^{+2}, K^{+} and Cl^{-1} influx and efflux. These changes result in the membrane depolarisation and upon changes in the cytosolic calcium levels, calmodulin (CAM) activates proteins through phosphorylation leading to

an increase in the intracellular metabolic cascade reaction (Radman *et al*., 2003, Nair, 2007).

In contrast, the bacterial hypothesised system, proposes a mechanism based on a cumulative effects rather than a single biological event (Murphy, 2008). Figure 1.8 represents a suggested mechanism of oligosaccharides elicitation in *B. licheniformis* culture. The suggested mechanism could include calcium and ROS as signalling molecules. Changes in calcium dependent enzymes, oligosaccharide-specific receptor (lectins) and protein kinases (enabling to phosphorylation of many proteins) might be required for an integral response, leading to a higher transcriptional activation of the secondary metabolite biosynthetic genes. Furthermore, it is suggested that calcium homeostasis might play an important role in the response mechanism, where changes in calcium levels could generate a variety of responses which activate the microbial defence system by recognition of the oligosaccharide-biotic elicitor.

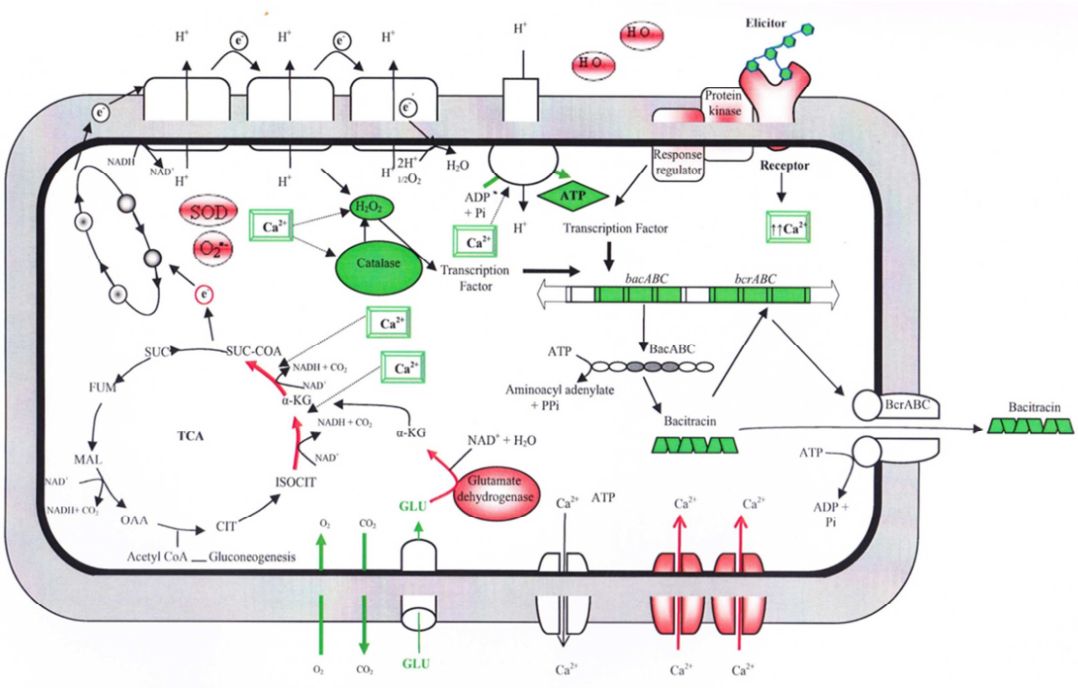


Figure 1.8 Proposed mechanism of elicitation in *B. licheniformis* in cultures for the production of bacitracin A (Murphy, 2008).

1.4 *Bacillus* spp. and their application

The genus *Bacillus* consists of a large number of diverse, rod-shaped aerobic and facultative anaerobic Gram-positive bacteria, they are motile due to the presence of flagella and capable of producing endospores that are resistant to adverse environmental conditions (Claus and Bekerley, 1986). Omnipresent in nature due to their distinct feature, they are capable of growing over a wide temperature range (30 ^{o}C to 45 ^{o}C), pH (2-11), (Sneath *et al*., 1986), and carbon and nitrogen sources (Veith *et al*., 2004).

Being able to form endospores, under favourable conditions, the vegetative cell cycle of the bacterium starts with the development of vegetative cells from endospores and the replications occurs via binary fission. Whenever the bacterium faces nutrient limitation or stress conditions, differentiation by the formation of sporangia occurs (Figure 1.9). Only one endospore is formed per cell and the spores are resistant to radiation, desiccation, cold and disinfectants (Fordyce, 1991). *Bacillus* species include free-living (e.g *B. subtilis*, *B. licheniformis*) and pathogenic species such as *B. anthracis.*

Bacillus spp. play an important role in the biotechnology industry since they are capable of producing wide range of metabolites such as enzymes, antibiotics, amino acids and vitamins (Rey *et al*., 2004). Most of them are extracellular metabolites; which facilitates the industrial downstream processing (Schallmey *et al*., 2004). Examples include ß-amylase and xylanases exploited in the paper industry (Ratanakhanokchai *et al*., 1999); glucanases which are extensively used in brewing (Tang *et al*., 2004) as much as proteases which are utilised for food-processing industries and food waste management (Cheng *et al*., 1995; Suntornsuk and Suntornsuk, 1995). Additionally to the extensive variety of enzymes, *Bacillus* species also produce vitamins (folic acid and riboflavins) used as additives in animal feed and human food industries (Sauer *et al*., 1998) as well as citric acids and amino acids (Rey *et al*., 2004).

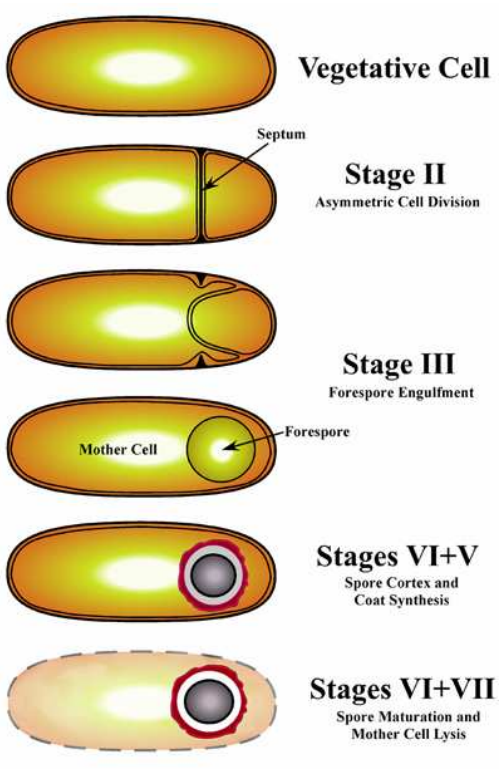


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Figure 1.9 *Bacillus* spp. spore formation.

In addition to the variety of metabolites, some species of *Bacillus* produce essential antibiotics, such as the antifungal mycobacillin and subtilin from *B. subtilis* (Banerjee and Bose, 1964), gramicin D and tyrocidine from *B. brevis* (Lipmann *et al*., 1971; Nakai *et al*., 2005), pumilin and tetain from *B. pumilis* (Katz and Demain, 1977), polymyxin from *B. polymyxin* polypeptin and circulin from *B. circulans* (Storm *et al*., 1977), thiocillin and cerexin from *B. cereus* (Shoji *et al*., 1976) and bacitracin and licheniformin from *B. licheniformis* (Johnson *et al*., 1945). Powerful biosurfactants can also added to the list; these include surfactins and lichenisins, reported to be involved in environmental applications such as in bioremediation (Joshi *et al*., 2008).

1.4.1 Bacitracin structure, properties and applications

Branched polypeptide with molecular weight of 1423, Bacitracin ($C_{68}H_{103}O_{16}N_{17}$) (Figure 1.10) is synthesised by *B. licheniformis* and *B. subtilis* strains (Johnson *et al*., 1945; Haavik, 1981).The first producing strain was isolated from a girl's wound in 1943 (Johnson *et al*., 1945). Currently, with improved biological activity, bacitracin is commercially available as zinc bacitracin.

Bacitracin is a mixture of similar polypeptides differing only by one amino acid (Figure 1.10). Bacitracins A and B are the most biologically active components (forming 95 % of biological activity), although analogue constituents are also synthesised during fermentation (B, C, D, E, and F). Bacitracin activity is pH dependent (better stability between pH 5-7), under alkaline conditions bacitracin A is oxidised to bacitracin F, an undesirable product with low antimicrobial activity (Konigsber *et al*., 1961). In contrast to many other antibiotics where the production starts at the stationary phase, bacitracin biosynthesis has been detected at early exponential phase. Hanlon and Hodges, (1981) suggest that early bacitracin production during growth helps the uptake of metal ions from soils poor in nutrients by changing the conformation of its membrane.

Bacitracin is widely used as an animal feed additive (Froyshov, 1984). The mechanism of domestic animal growth promotion by bacitracin is unknown. As feed additives, zinc bacitracin has demonstrated to improve animal growth and production by causing cell wall lysis of the intestinal bacterium such as *E. coli* (Butaye *et al*., 2003).

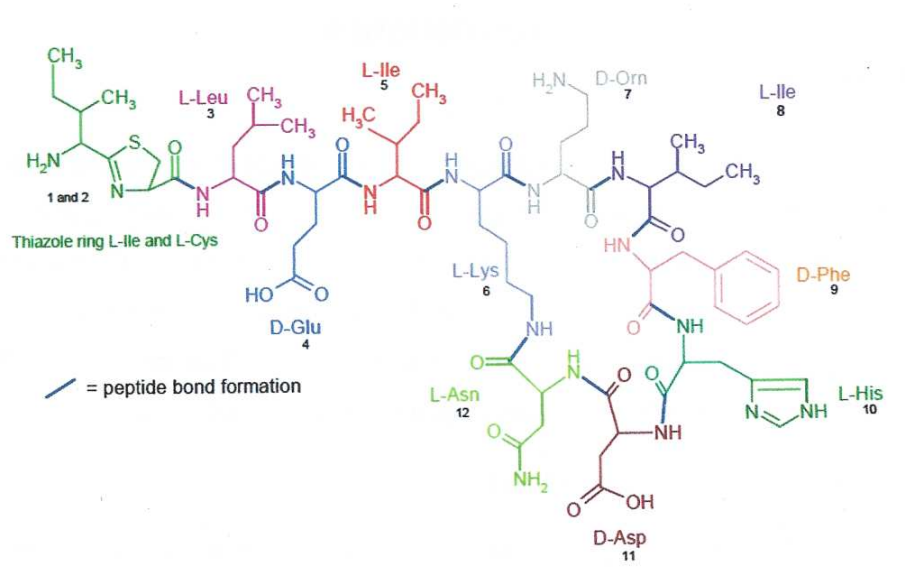


Figure 1.10 Bacitracin A chemical structure. The figure shows the thiazole ring formed from L-isoleucine and L-cysteine and the cyclic heptapeptide ring formed by a reaction of the amino group of L-lysine with the β-carboxyl group of D-asparagine (Newton and Abraham, 1953).

In the pharmaceutical industry, bacitracin is largely used in creams, ointments, antibiotic sprays and powder, mostly combined with other antibiotics such as neomycin and polymyxin B. This enhances bacitracin effect against all bacterial species (Kucers, 1997). Bacitracin has microbial activity mainly against Gram-positive bacteria such as *Staphylococcus*, *Streptococcus* and *Clostridium* (Johnson *et al*., 1945; Hickey, 1964), where it inhibits the dephosphorylation of lipid pyrophosphate, a process occurring during the bacterial cell wall synthesis (Stone and Strominger, 1971).

1.4.2 Bacitracin biosynthesis

Bacitracin is synthesised by a multi-enzyme complex described as bacitracin synthetase ABC (Froyshov, 1977) under non-ribosomal multiple carrier model. This model was proposed in 1996, when Stein and co-workers demonstrated that each synthetase enzyme has modules and each module carried one cofactor (phosphopantetheinic acid presented as a phosphopantethene arm - 4'PAN cofactor).

Figure 1.11 represents the bacitracin biosynthesis carried out by a multiple carrier model. Enzyme A contains sites for 1-5 amino acids: isoleucine, cysteine, leucine, glutamic acid and isoleucine. Enzyme B activates amino acids 6-7 such as lysine and ornithine and enzyme C activates 8-12 amino acids: isoleucine, phenylalanine, histidine, aspartic acid and asparagine.

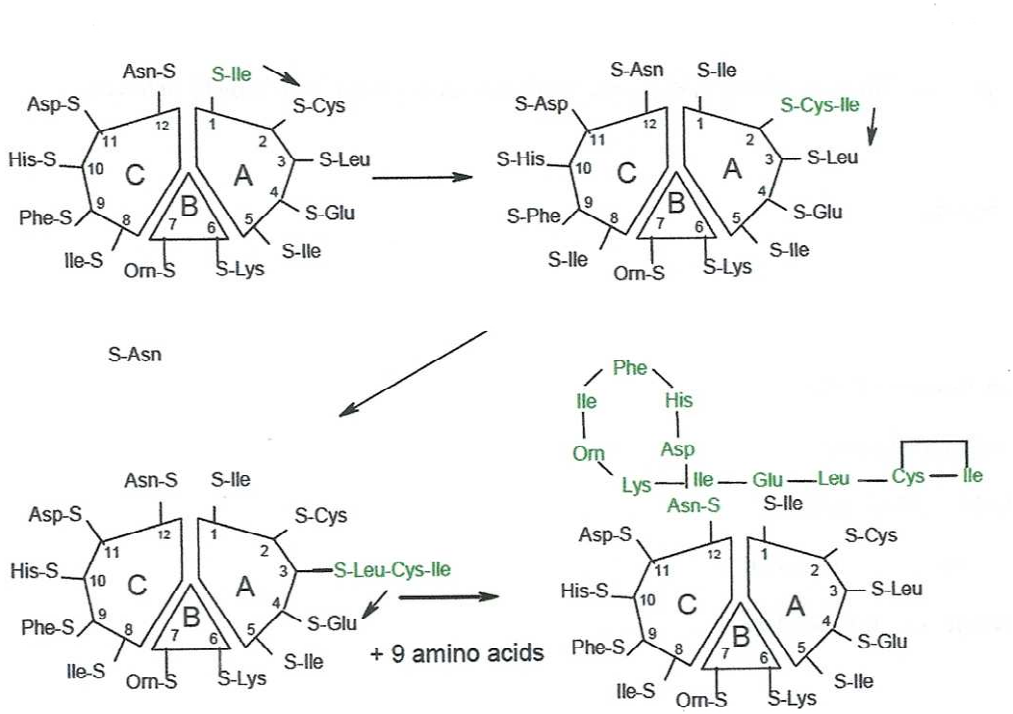


Figure 1.11 Bacitracin biosynthesis by the multiple carrier model. Each enzyme subunit carries modules and each module carriers its own 4' PAN cofactor (Stein *et al.*, 1996).

Bacitracin synthesis is initiated by the amino group of L-isoleucine to which the other amino acids are added consecutively in the N (amino end) to C-end (carboxyl-end) direction. Biosynthesis starts by the activation of the amino acids by the ATP hydrolysis, resulting in aminoacyl adenylates and pyrophosphate, the latter served as energy source (Froyshov, 1984). The aminoacyl adenylate (L-isoleucine) binds to the 4'PAN cofactor by forming a thioester bond (Froyshov, 1977). At this stage the next activated amino acid (cysteine) is bound to the enzyme through the second 4'PAN cofactor. The first 4'PAN cofactor moves closer to the second 4'PAN cofactor carrying cysteine and the dipeptide is formed liberating the first 4 'PAN cofactor. This is repeated for the next amino

acid. Once the pentapeptide is formed, the chain is transferred onto enzyme B. When two more amino acids are attached, the polypeptide passes to enzyme C (Froyshov, 1977).

Once the dodecapeptide is formed, the carboxyl group of the asparagine reacts with the amino group of lysine forming a heptapeptide ring by an amide bond and at the same time releasing the antibiotic from the enzyme complex (Froyshov, 1977). The catalytic functions of the enzyme are related to the organisation of their biosynthetic genes. The multienzyme complex in charge of bacitracin production as described above is composed of bacitracin A, B and C peptide synthetases, which are encoded by the bacitracin biosynthetic genes *bacA*, *bacB* and *bacC* (Eppelmann *et al*., 2001).

1.5 β-mannanase

From the glycoside hydrolases family, mannan endo-1,4-β-mannosidase or 1,4-β-D-mannanase (EC 3.2.1.78), is commonly named as β-mannanase. β-mannanase is an enzyme that catalyses random hydrolysis of β-1, 4-mannosidic linkages in mannan-oligosaccharides (MO) and mannan-based polysaccharides, such as glucomannas and galactomannas. The β-mannanase mode of action differ from its source and the substrate utilised (Bo *et al*., 2009), but commonly as result of a multi-step reaction, mannose is generated from the degradation of MO (Figure 1.12) whereas as a result of the enzymatic degradation of heteromannas, manno-oligosaccharides with a DP ranging from 2 to 10 are generally formed (Bo *et al*., 2009; Songsiriritthigul *et al*., 2010).

Mannanses are reported to be ubiquitous in nature, been isolated from a wide variety of organism, including bacteria, actinomycetes, yeasts, fungi, plants and animals (Dhawan and Kaur, 2007). They have been isolated and characterised from several fungal species such as *Aspergillus tamarri* (Civas *et al*., 1984), *Sporotrichum cellulophilum* (Araujo and Ward, 1990), *Trichoderma reesei* (Stalbrand *et al*., 1993) and *Penicillium oxalicum* (Kurakake *et al*., 2006). In higher plants, some examples are reported; *Arabidopsis* spp. (Yuan *et al*., 2006c), *Sebasnia virgate* (Lisboa *et al*., 2006), *Lycopersicon esculetum Mill* (Stiel and

Bradford, 1997), Sesame (Carvalho *et al*., 2001) and seeds of *Lactuca sativa* (Nascimento *et al*., 2004).

In prokaryotes, Gram-positive bacteria are reported to be the main source of β-mannanase. Among them, several *Bacillus* species (Sun *et al*., 2003), including *B. licheniformis* (Feng *et al*., 2003; Songsiriritthigul *et al*., 2010) and *B. subtilis* strains (Tian *et al*., 1993) are stated. C*lostridia* (Kataoka and Tokiwa, 1998; Perret *et al*., 2004), and actinomycetes species (Kansoh and Nagieb, 2004), are also described as mannan degraders. Additionally, few Gram-negative strains are also reported; *Pseudomonas* (Braithwaite *et al*., 1995) and *viz. Vibrio* (Tamaru *et al*., 1997).

In microbial system, β-mannanase enzyme is normally secreted extracellularly into the medium in which the microorganism is cultured (Dhawan and Kaur, 2007). ß-mannanase biosynthesis can be either constitutive or induced in the presence of mannan and galactomannas (Araujo and Ward, 1990; Ademark *et al*., 1998).

A significant number of applications for β-mannanase are reported in literature. In pulp and paper industries, mannanase have shown to be effective in increasing the brightness of pulps in bleaching experiments, most notably in combination with xylanases (Clarke *et al*., 2000). In the food and feed industries, it is used in the production of fruit juices and soluble coffee (Gomes and Steiner, 1998; Nicolas *et al*., 1998) as well as in the preparation of poultry diets (Daskiram *et al*., 2004). β-mannanase has also been shown to have a strong potential as viscosity reducers of hydraulic fracturing fluids used in oil and gas production (Adams and Kelly, 1995).

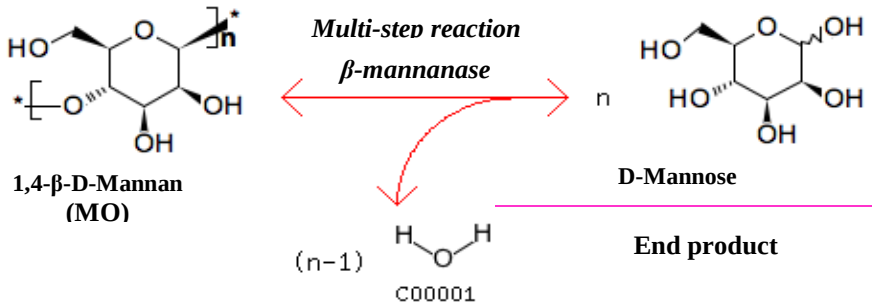


Figure 1.12 β-mannanase MO degradation.

1.6 Intracellular calcium

Microorganisms have developed sophisticated signalling pathways to deal with dramatic environmental changes such as oxidative, osmotic, pH and nutrient limitation stresses (abiotic stress) (Lopez-Maury *et al*., 2008) or the addition of elicitors such as oligosaccharide, protein and lipids (biotic stress) that may or not affect their normal growth.

Facing the environmental challenge, microorganisms reprogram their cellular set up by triggering a network of signalling events that start with signal perception at the membrane level and ends with a cellular response (defence mechanism) (Tuteja and Mahajan, 2007). The extracellular stress signal transduces inside the nucleus to induce multiple stress responsive genes, the products of which ultimately lead to microbial adaptation to stress tolerance directly or indirectly (Apel and Hirt, 2004).

Signal transduction requires a temporal coordination of all signalling molecules. In eukaryotes systems, many molecules acting as second messenger in signalling pathways have been reported; these include Ca^{2+}, lipids like inositol triphosphate (IP_3) and cyclic guanosine monophosphate (cyclic GMP) (Tuteja and Mahajan, 2007).

Studies investigating signal transduction in abiotic and biotic stress in plant systems, revealed that changes in cytosolic Ca^{+2} across the cell membrane was able to regulate a range of chemical signals through different intracellular

signalling pathways, resulting in a variety of cellular reactions (White and Broadley, 2003; Nelson *et al*., 2004). This is possible due to the presence of efficient calcium efflux (primary and secondary antiporters) and influx (voltage-gated calcium channels) systems and the existence of calcium binding proteins (calmodulin) which are capable of maintaining a balanced homeostasis across the cell membrane (Murphy, 2008). Calmodulin is a calcium dependent protein which changes conformation upon calcium binding, resulting in its activation. Calmodulin can interact with more than 25 proteins such as phosphatases and kinases and modify their activity (Norris *et al*., 1996).

Hence, in order to maintain calcium homeostasis, calcium channel and calcium transport systems must be present in the system (Dominguez, 2004). Evidence of the existence of voltage operated calcium channels (VOCCs), primary/secondary calcium transport systems as well as calmodulin-like proteins have been reported in prokaryotes system (Norris *et al*., 1996) including in *B. subtillis, S. erythraea*, Cyanobacteria, and *Mycococcus xanthus* (Michiels *et al*., 2002).

In *Bacillus* spp. VOCCs of protein nature have been found to be responsible for the influx of calcium. This type of VOCCs displays similar characteristics such as voltage dependence and selectivity for divalent ions as present in eukaryotes (Noris *et al*., 1996).

Bacterial efflux mechanism has been classified into two main categories: primary exchanger which can lead to calcium secretion at the expense of ATP, examples include ATPases (Herbaud *et al*., 1998) and secondary exchangers (Ca^{2+}/H^{+} and Na^{+}/Ca^{2+}) which are powered by electrochemical gradients of protons and sodium ions (Dominguez 2004, Shemarova and Nesterov, 2005). Schematic representation of calcium transport system and its role in cell transduction is shown in Figure 1.13.

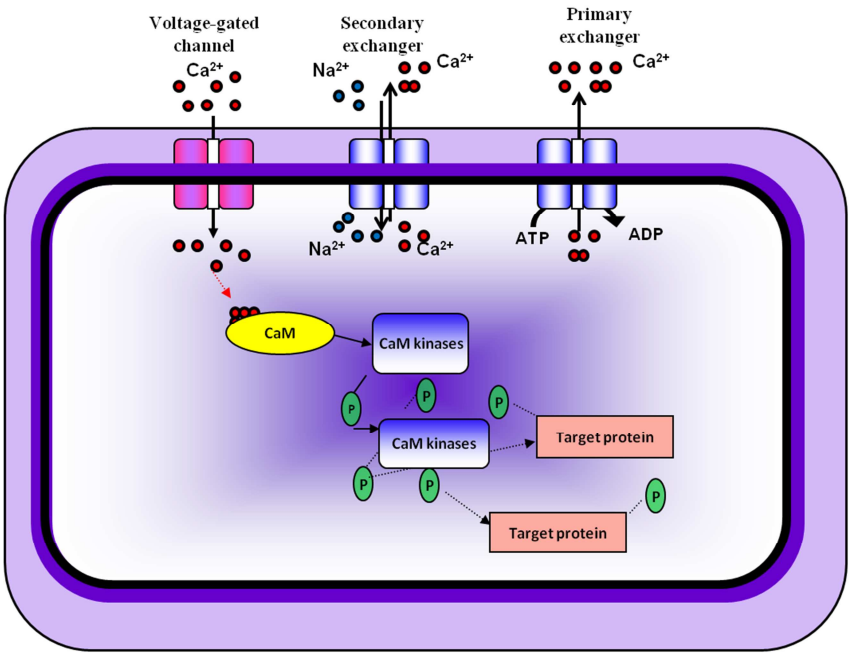


Figure 1.13 Schematic representation of calcium transport system and its role in cell signalling.

In general, Ca^{+2} play an important role in cellular processes, being able to generate cascade reactions through the regulation and activation of signal transduction (Norris *et al*., 1996; Holland *et al*., 1999; Dominguez, 2004). Changes in Ca^{+2} concentrations have been reported upon addition of stimuli in bacterial cell cultures (Campbell, 1983; Norris *et al*., 1996; Murphy *et al*., 2011). The role of calcium in bacterial cultures has been recently recognised as a secondary messenger, where calcium alterations are sensed by calcium binding proteins. Calcium binding proteins through activation/deactivation of numerous enzymes transmit information resulting in a physiological response. In addition, Ca^{+2} has been suggested to be involved in prokaryotic cell division, chemotaxis, motility, competence, sporulation, cell defence, gene expression and stress signals (Watkins *et al*., 1995).

Biotic-elicitation studies in bacterial cultures have indicated that oligosaccharide-elicitors are able to enhance Ca^{+2} concentrations in *E. coli* and *B. subtilis* after there to the bacterial cells (Murphy *et al*., 2011).

1.6.1 Measurement methods

Measurements of intracellular calcium in prokaryotes have been possible due to the advent of specific calcium probes such as aequorin, a protein that emits luminescence upon calcium binding (Cobbold *et al*., 1991), and by specific fluorogenic compounds that specifically bind to calcium (ions probes widely used as intracellular indicator). In this case, calcium ion detection is most often accomplished by using a dye that has a recognition portion as well as a region that confers fluorescence (Nair, 2007).

Calcium indicator dyes are classified into two groups; the ratiometric dyes that have different excitation or emission wavelengths in the presence of calcium than they have in its absence, examples are Fura-2 and Indo-1 (Haughland, 2002), and non-ratiometric dyes, the ones that increase their fluorescence in the presence of calcium without changing wavelengths. Examples include: calcium orange, Fluo-3 and Fluo-4.

1.6.1.1 Fluo-4 cell loading

Several approaches have been developed for loading fluorescent ion indicators into the cell. However, the most commonly used method is acetoxymethyl (AM) ester loading (Figure 1.14). This non-invasive technique uses compounds where the carboxylates groups of the indicator dyes have been derivated as acetoxymethyl esters resulting in a nonpolar compound that is permeable to cell membranes. Once inside the cells, these compounds are hydrolysed by intracellular esterases. Once acted upon by esterases, the resultant activated indicator is now a polar molecule that is no longer capable of freely diffusing through the cell membrane, essentially trapping the compounds inside the cell (Figure 1.14) (Hibbs, 2004).

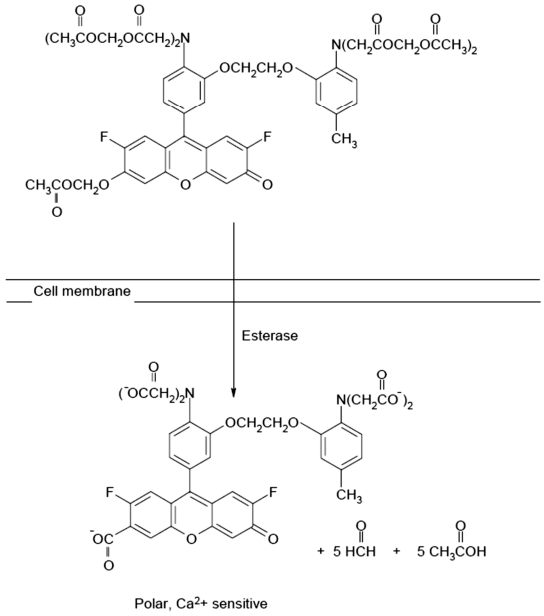


Figure 1.14 Schematic diagram of the processes involved in loading cells using membrane-permeant acetoxytomethyl (AM) ester derivatives of fluorescent indicators, in this case Fluo-4 (Nair, 2007).

Although changes in Ca^{2+} levels in bacteria under stress conditions have been previously described (Murphy *et al*., 2011), there are no reports to date regarding the effects of oligosaccharide elicitors OG, OM and MO on the intracellular Ca^{2+} levels and its role on bacitracin A production in *B. licheniformis* cultures.

1.7 Proteomics

Proteomics is a powerful tool for analysing differences in protein expression occurring in living systems as a response to external stimuli (Teixeira *et al*., 2005; Bhadauria *et al*., 2009). Several proteomic methods have been developed for assessment of microbial response to stress; however, the method employed may differ from species to species.

In a two dimensional electrophoresis system, (Figure 1.15), subsequent to protein extraction, protein samples are firstly separated by their isoelectric point (pI) during isoelectric focusing (IEF: first dimension) and secondly based on molecular mass during sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE: second dimension) (O'Farrell, 1975). The resulting

profile allows the detection of proteins of interest, which are identified by mass spectrometry and by sequence matching on the NCBI database.

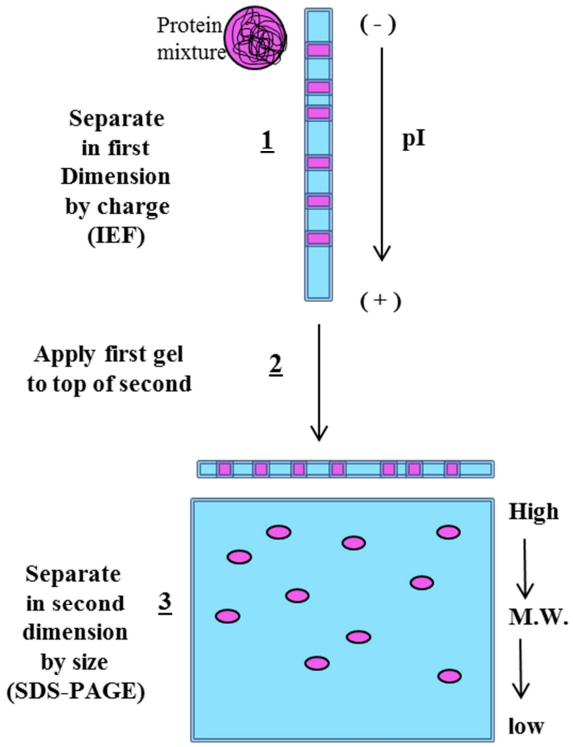


Figure 1.15 Schematic 2-D electrophoresis processes.

1.7.1 Protein phosphorylation

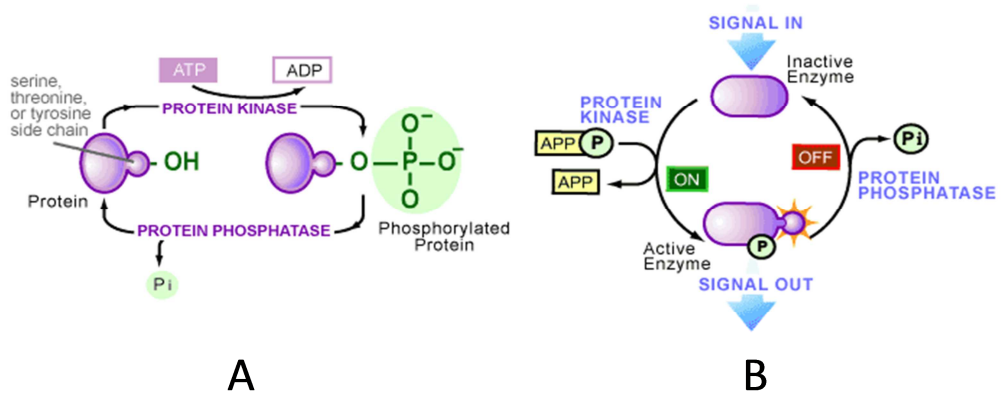
A rapid microbial metabolic adaptation to environmental stress is essential for microbes to survive adverse conditions. Microorganisms have developed numerous signal-transduction pathways involving receptors receiving signals that are transmitted to intracellular entities. Reversible phosphorylation of proteins is an evolutionary conserved regulatory mechanism, essential for enzymatic and metabolic regulation in living systems (Lomas-Lopez *et al*., 2007).

The process involves specific protein kinases coupled to dephosphorylation reactions carried out by protein phosphatases (Hunter and Plowman, 1997). Protein kinases phosphorylate substrate proteins, by taking a phosphate off the chemical energy-carrying molecule ATP and place it onto an amino acid side chain of a protein (Figure 1.16 A). The hydroxyl group (-OH) of serine, threonine,

tyrosine or histidine amino acid side chains is the most common target. The high charge density of phosphate causes a potent perturbation in protein structure and function. This has a great effect on the protein catalytic activity, location within the cell and affinity for a ligand. It has been reported that reversible phosphorylation/dephosphorylation regulates enzymes catalytic action by speeding it up or slowing it down (Saez, 2005) (Figure 1.16 B).

As a member of one of the largest families of regulatory enzymes, protein kinases (Martin *et al*., 2003) can be categorised according to the amino acid species they phosphorylate. Examples include serine/threonine kinase, tyrosine kinases and histidine kinases. There are several serine/threonine kinases that function in signal transduction pathways. The more commonly known are cAMP-dependent protein kinase (PKA), calmodulin dependent protein kinase (PKC) and the mitogen activated protein kinases (MAPK) (Wurgler-Murphy and Saito, 1997).

Reversible phosphorylation of serine/threonine/tyrosine residues has been established in bacteria, both in Gram-negative and Gram-positive species (Vicent *et al*., 2000; Deutscher and Saier, 2005) and it has been found to be involved in metabolic control of sporulation and stress responses (Duncan and Hershey, 1989; Balodimos *et al*., 1990; Madec *et al*., 2002; Rajagopal *et al*., 2006).



http://www.scq.ubc.ca

Figure 1.16 Role of protein kinase and protein phosphatase on phosphorylation of proteins. **(A):** Addition of a phosphate group to an amino acid and, **(B):** biological activation of the target protein by the phosphorylation.

1.8 Batch and Continuous Fermentation

Batch fermentation refers to a closed system in which the nutrients required for the microbial growth are added into a fermenter, decontaminated before the process starts and then, removed at the end of the fermentation process. In this mode of operation, microbial cells are continuously changing with time, for this reason batch fermentation is considered as an unsteady-state system (Stanbury and Whitaker, 1999). Nevertheless, continuous fermentation is an open system. Sterile nutrient solution is added to the bioreactor continuously and an equivalent amount of converted nutrient solution with microorganisms is simultaneously removed from the system (Stanbury and Whitaker, 1999).

A continuous culture system starts as a batch culture, where microbial growth proceeds under familiar growth cycle. The addition of fresh medium to the culture at a particular rate during the exponential growth, follow by the removal of the culture medium at a suitable rate allows growth to precede rate indefinitely (Figure 1.17). As a result of this, the microbial population within the culture can grow at a constant rate in a constant environment and assumes a steady state (Hoskisson and Hobbs, 2005).

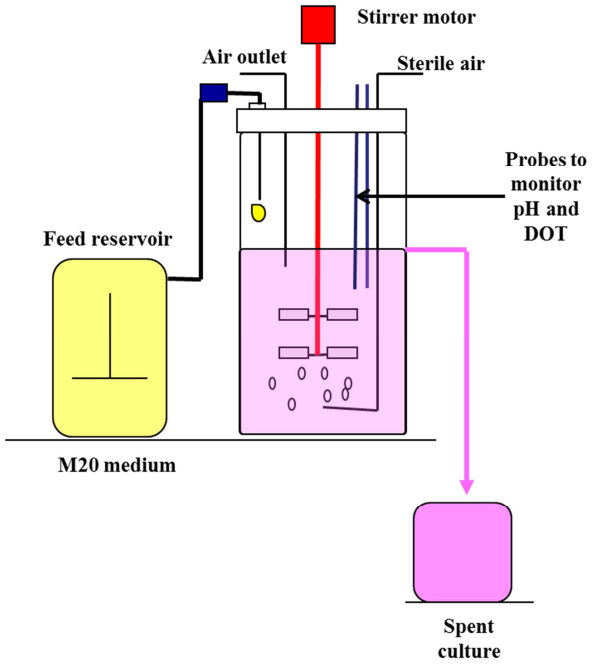


Figure 1.17 Schematic representation of a continuous culture process.

A continuous culture system is employed when steady state microbial culture is required. The chemostat theory was first presented by Monod (1942), when the author described the relationship between specific growth rate (μ) of a microbial population and the substrate concentration (s). The author showed that the progression event of a growth cycle (lag, exponential and stationary phase) is not an intrinsic property of the organism but a result of its interaction with the physico-chemical environment in which it is growing (Tempest, 1969).

The continuous culture developed by Monod, showed that the influx of medium from a reservoir is balanced by the efflux of culture broth, hence allowing growth to occur under steady state, with growth of new cells being balanced by those washed out. Thus the growth of new biomass is equal to the rate at which the culture is being diluted. Hence, in steady state system, the growth rate can be manipulated as a function of the dilution rate (Hoskisson and Hobbs, 2005).

The advantage of such system is that bacterial growth can be manipulated to achieve slow growth rates, which more closely resemble those found in nature (Drake and Brogden, 2002). By doing so, studies have shown that bacteria grown under these conditions express cell wall proteins and respond to environmental stimuli much like cells *in vivo*. Continuous culture has been adopted to study microbial communication and to understand how microbial communities respond to external stimuli (Bradshaw and Marsh, 1994; Kinniment *et al*., 1996b). Bacon *et al*., (2010) applied continuous culture system for measuring the environmental stress on mutation of *Mycobacterium tuberculosis* whereas in *Streptococcus mutans* and *Entercoccus hirae* continuous culture showed the bacteria adaptation process to acid stress (Belli and Marquis, 1991). Continuous culture has been widely used in order to study *E. coli* adaptation process to succinate stress (Kwon *et al*., 2011), UV radiation (Berney *et al*., 2007) and osmotic stress (Welsh and Herbert, 1993). Continuous culture has been also applied to investigate the physiological response of *Bacillus* spp. during starvation (Silva *et al*., 2005).

Chapter 2

2.0 Materials and Methods

2.1 Materials

All materials used in this study were purchased from Sigma Chemical Co (Poole, Dorset, UK) unless stated otherwise. For quantitative and qualitative assays, analytical grade reagents and ultra-pure water were used.

2.2 Microorganism

Bacillus licheniformis NCIMB 8874 was purchased from the National Collection of Industrial and Marine Bacteria, USA.

2.2.1 Medium for maintenance and growth of *Bacillus licheniformis* and bacitracin production (M20)

Nutrient agar was used for maintenance of bacterial slants. Chemically defined medium (Table 2.1) was used for the growth of *B. licheniformis* cultures and production of bacitracin A. All solutions were prepared with distilled water and autoclaved at 121^{o}C for 15 min apart from L-glutamic acid and ferrous sulphate heptahydrate, which were filter-sterilised (using a 0.2 μm syringe filter purchased from Orange Scientific) and added separately. The pH of the medium was adjusted to 6.0 with 4M NaOH prior to sterilisation.

Table 2.1: M20 defined medium composition.

Compound	(g L^{-1})
L-Glutamic Acid	20.00
$NaH_2PO_4 \cdot 2H_2O$	20.00
Citric Acid	1.00
Na_2SO_4	0.50
KCl	0.50
$MgCl_2 \cdot 6\ H_2O$	0.20
$Ca\ Cl_2 \cdot 2\ H_2O$	0.01
$Mn\ SO_4 \cdot\ H_2O$	0.01
$Fe\ SO_4 \cdot 7\ H_2O$	0.01

2.3 Fermentation conditions

Shake Flasks (SF) 500 mL, without baffles were used for all the experiments unless stated otherwise. FerMac 360, Electrolab Ltd UK, 0.6-L and 2.5-L Stirred Tank Reactors (STR) were used for further elicitation studies. Fermenters with identical design and equal working volumes (1.5 L) were used to carry out parallel batch fermentation using different conditions. The 0.6-L STR was used for continuous culture experiment with 0.3 L working volume.

2.3.1 Shaken Flasks and Bioreactor Fermentations

Stock cultures on agar slants were sporulated for 7 days at 37 ^{o}C and stored at 4 ^{o}C. Growth medium was inoculated in SF with 1 mL of spore suspension (10^{7} spores mL^{-1}) and incubated at 37 ^{o}C on a rotary shaker at 200 rpm for 16 hrs.

For SF fermentation, 10 mL (10 % of the working volume) of 16 hrs old inoculum was transferred into a 500 mL flask containing 90 mL of M20 medium. Incubation was carried out at 37 ^{o}C on a rotary shaker at 200 rpm (2 cm-throw) for 72 hrs. SF studies were carried out in triplicate.

For STR fermentation, 0.6-L and 2.5-L fermenters were sterilised for 40 min at 121 ^{o}C, containing M20 medium. The fermenters were inoculated (10 % of the STRs working volume) with an aerobic overnight preculture of *B. licheniformis*. The temperature was maintained at 37 ^{o}C. The air inlet flow-rate was 1 vvm and the stirrer speed was changed controlled to maintain the dissolved oxygen tension (DOT) above 30 % air saturation. Foaming was controlled by adding 0.5 % (v/v) sterile Antifoam 204 (Sigma). There were no differences in the agitation rates between the control and elicited fermenters.

For continuous culture, *B. licheniformis* cells were grown under batch mode up to OD ~ 2.5 (650 nm) after which M20 medium was continuously pumped into the fermenter (F = 0.40 mL min^{-1}, calculated based on the μ = specific growth rate). The culture was kept under the continuous mode for three volume changes in order to obtain a consistent growth. Thereafter, the continuous culture was run

with the test tank (FT) containing M20 medium supplemented with 300 mg L^{-1} MO and the control tank (FC) containing medium M20 as seen in Figure 2.1.

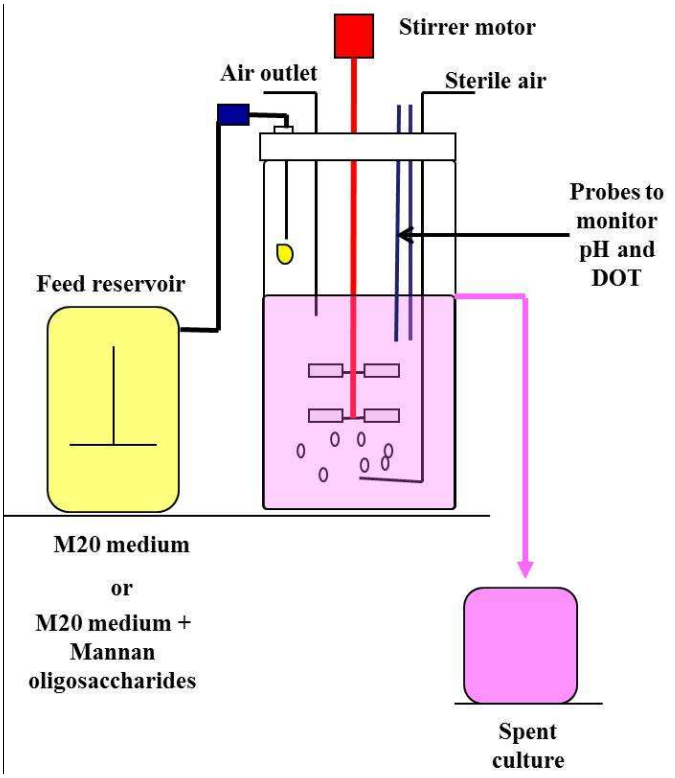


Figure 2.1 Continuous culture diagram of *B. licheniformis.*

2.4 Preparation of elicitors

2.4.1 Mannan oligosaccharides (MO) elicitors from locust bean gum

MO was prepared by enzymatic hydrolysis of Locust Bean Gum (LBG) as described before (Asilonu *et al*., 2000). One gram of LBG was dissolved in 50 mL of distilled water and heated at 80 ^{o}C. One hundred microliters of gamanase enzyme (Novozyme, Denmark) was added to the mixture. The reaction was carried out for exactly five minutes and quenched by increasing the temperature to 100 ^{o}C. The mixture was cooled down to room temperature and the top layer (soluble MO) was frozen at -80 ^{o}C. The frozen mixture was then freeze-dried and stored at 4 ^{o}C. The same prepared batch of MO has been used for all the experiments carried out in this thesis.

2.4.2 Oligoguluronate and Oligomannuronate elicitors from sodium alginate

Oligoguluronate (OG) and Oligomannuronate (OM) were prepared by partial acid hydrolysis of sodium alginate (Asilonu *et al*., 2000). Ten grams of sodium alginate were dissolved in 500 mL of distilled water by heating and agitation. On complete dissolution 500 mL of warm 0.6 M hydrochloric acid was added. The solution was refluxed at 100 ^{o}C for six hours, rapidly cooled down to room temperature and centrifuged at 4600 rpm (Sorvall legend RT, Thermo Scientific) for 30 min. The supernatant was discarded and the precipitate was rinsed and re-suspended in 300 mL of distilled water. Sodium hydroxide (0.3 M) was added until all solid particles were dissolved. Sodium chloride was then added to make a final concentration of 0.5 % (w/v). An equal volume of ethanol (99 %) was added to the solution and allowed to stand overnight. The solution was centrifuged, the supernatant discarded and the precipitate was rinsed and re-suspended with 200 mL of distilled water. The pH was adjusted to 2.8 with 0.3 M of hydrochloric acid and centrifuged at 4,600 rpm (Sorvall legend RT, Thermo Scientific) for 30 min. The supernatant (OM) and precipitate (OG) were separated, freeze-dried and stored at 4 ^{o}C. The same prepared batch of OG and OM has been used for all the experiments carried out in this thesis.

2.4.3 Elicitation conditions

Oligosaccharide elicitors were dissolved in distilled water, (in concentrations ranging from 100 to 300 mg L^{-1}) and sterilised at 121 ^{o}C for 15 min. The elicitors were added aseptically to the production culture at 0 hrs and/or 24 hrs after inoculation. For the effect of elicitors' concentration studies, an experimental set up was designed (Table 2.2). No elicitor was added to the control cultures.

Table 2.2: Experimental Design for Bacitracin A production in SF.

Run	Elicitor	(mg L^{-1})	Addition time (hrs)
C 1	-	-	-
T 1	MO	25	24
T 2	OG	25	24
T 3	OM	25	24
T 4	MO	50	24
T 5	OG	50	24
T 6	OM	50	24
T 7	MO	500	24
T 8	OG	500	24
T 9	OM	500	24
T 10	MO	1000	24
T 11	OG	1000	24
T 12	OM	1000	24

2.5 Assay procedures

2.5.1 Optical density, pH measurements and cell dry weight assay

The optical density (OD) of the fermentation samples was read at 650 nm, where chemically defined medium was used as a blank. Absorbance readings above 0.5 were diluted with M20. The pH was also monitored. Cell dry weight (CDW) was calculated from the measured OD value according to a linear relationship between the CDW and the OD of the culture. CDW and OD correlation was determined in previous studies as seen in the Appendix 1. Estimation of cell CDW from the optical density of the culture was needed in order to be able to calculate the bacitracin yield produced by the culture at specific time.

2.5.2 Bacitracin Assay

The concentration of bacitracin A was quantified using a HPLC-based method (Pavli and Kmetec, 2001). The gradient elution system consisted of a C8 (5µ), 150 x 4.6 mm Kromasyl reverse phase column (Phenomenex) maintained at 40 ^{o}C, where the flow rate of the two mobile phases was set to 1.4 mL min^{-1} and an injection volume of 20 µL (Tables 2.3 and 2.4). Bacitracin was detected under UV light at 254 nm. Zinc bacitracin (Sigma, UK) was used as a standard. A calibration curve (R^2 = 0.9999) was constructed using bacitracin concentration ranging from 0-1000 mg L^{-1} (see appendix 2).

Table 2.3: Mobile phase composition of Bacitracin A detection.

Mobile phase	Methanol (mL^{-1})	Acetonitrile (mL^{-1})	KH_2PO_4 (mL^{-1})
Buffer A	245	245	510
Buffer B	300	300	400

Table 2.4: HPLC gradient profile for analysis of Bacitracin A.

Sector	Time (min)	Percentage Buffer A	Percentage Buffer B
1	0-12 min	100 %	0
2	12-18 min	0	100 %
3	18-20 min	100 %	0

2.5.3 Detection of Mannan oligosaccharides by thin layer chromatography (TLC)

Mannan oligosaccharides profile and fate during the growth of *B. licheniformis* were determined by TLC. TLC of all purified oligosaccharides was carried out on silica gel 60 plates (Merck, Germany). The adsorbent thickness of the plates was one millimetre. The chromatography solvent contained: water, 20 %; ethanol, 30 %; and butan-1-ol, 50 %. The solvent (150 mL) was prepared in a five-litre (25 x 8 x 25 cm) TLC tank and left closed for 1 hrs to saturate the tank before use. Equal volumes (30 µl) of oligosaccharides and known standards (1 g L^{-1}) were applied on the TLC plates at horizontal 15 mm intervals and air-dried. After drying, the plates were placed in the solvent and allowed to run for 5 hrs. Plates were then removed from the solvent, air dried and sprayed with identifying reagent. The identifying reagent contained a mixture of 0.2 % naphthoresorcinol (Sigma) (w/v) and 0.4 % diphenylamine (Sigma) (w/v) in ethanol (96 mL) and 4 mL of concentrated sulphuric acid (added before use). Sprayed plates were allowed to dry for 5 min and then were placed in a 100 °C oven for 10 to 15 min to develop the characteristic TLC spots used for the identification of the oligosaccharides. Mannose, Mannobiose, Mannotriose and Mannohexaose (Megazyme) were used as standard.

2.5.4 Profile determination of MO by High performance anion exchange chromatography (HPAEC)

The prepared elicitors (OG, OM and MO) were analysed by HPAEC-based method with pulsed amperometric detection. The gradient elution system consisted of a Carbo Pac PA 200 (Dionex) maintained at 19 ^{o}C, where the flow rate of the two mobile phases (Table 2.5 and 2.6) was set to 0.5 mL min^{-1}. Mannose, Mannobiose, Mannotriose and Mannohexaose (Megazyme) were used as standard.

Table 2.5: Mobile phase composition for MO detection.

Mobile phase	NaOH (M)	NaAC (M)
Buffer A	0.1	1
Buffer B	0.1	0.05

Table 2.6: HPAEC profile for analysis of MO.

Time (min)	Buffer A %	Buffer B %
0-19	5	95
20-30	20	80
31-45	25	75
45.1-60	5	95

2.5.5 Total carbohydrate assay

Total carbohydrate assay was carried out as described by Chaplin and Kennedy, (1994). A standard calibration curve was constructed using glucose, OG and MO ranging from 0-1000 mg L^{-1}. For this assay, 200 µL of samples, standard or blank were added to 200 µL of 5 % phenol. Sulphuric acid (1 mL) was rapidly added to the solution and allowed to stand undisturbed for 10 min. The mixture was then mixed and allowed to stand for further 30 min. Absorbance measurements were read at 490 nm. Samples were analysed in triplicate.

2.5.6 Reactive oxygen species

The effect of different carbohydrate-based elicitors on Reactive oxygen species (ROS) was investigated in *B. licheniformis* cultures. Determination of ROS was carried out as described by Murphy, (2008). One milligram of 2'7'-Dichlorodihydroflurescein diacetate, DCFH-DA (Sigma-UK) was dissolved in 1 mL of ethanol (HPLC grade). The dissolved DCFH-DA was then added to 499 mL of salt solution (M20 medium in the absence of L-glutamic acid and citric acid) to achieve a final concentration of 2 mg L^{-1}.

Dynatech 'white clear bottom' 96 microwell plates were used in this study. Each well contained 30 µL of oligosaccharides, 130 µL of *B. licheniformis* culture and 130 µL DCFH-DA solution. FLUOstar Optima (BGM, UK) was used to measure the fluorescence. Fluorescence was recorded every 5 min for 30 min at 485 nm excitation and 530 nm emission.

2.5.7 Superoxide dismutase

The SOD activity of fermentation samples (control and elicited cultures) were measured using superoxide dismutase kit from Sigma. SOD activity assay was carried out following the manufacturer's protocol. Two millilitres of the samples were harvested by centrifugation at 13,000 rpm (Bio fresco, Heraeus) for 10 min at 4 ^{o}C. The pellets were added to each FastPrep blue (MP biomedical) tube containing the lysing matrix and lysing buffer (20 mM Tris pH 8). These mixtures were shaken in the Fastprep instrument at a speed of 6.5 ms^{-1} for 45 s. Experiments were carried out in triplicate.

2.5.8 Catalase assay

The catalase activity of the fermentation samples was measured and compared. Two millilitres of the cultures was harvest by centrifugation at 13,000 rpm (Biofreasco, Heraeus) for 10 min at 4 ^{o}C. The cells were washed twice with 10 mM of potassium phosphate buffer containing 1 mM of phenylmethysulfonyl fluoride dissolved in ethanol. The cells were added to each FastPrep blue tube

(MP biomedical) and then mixed in the Fastprep instrument at a speed of 6.5 ms^{-1} for 45 s.

Catalase activity was determined as described by Beers and Sizer (1952) where the rate of disappearance of H_2O_2 (3 %) was measured spectrophotometrically at 240 nm using a quartz cuvette (1 cm path length). Twelve microliters of 3 % H_2O_2 were added to the supernatant in a total volume of 1 mL and the reaction was measured every second for 30 s at 30 ^oC. One unit of enzyme was expressed as micromoles of O_2 produced from the degradation of 1 µmol H_2O_2 per minute per millilitre of the sample at 30 ^oC. Experiments were carried out in triplicate.

2.5.9 β-mannanase enzyme activity

The activity of β-mannanase was determined using azo-carob galactomannans dyed with Remazol Brilliant Blue R (Sigma) (10 g L^{-1}) as substrate. A reaction mixture containing 0.9 mL substrate in phosphate buffer (50 mM pH 7) and 0.1 mL of the diluted enzyme solution was incubated at 50 ^oC for 10 min. The reaction was stopped by adding 2 volume (v/v) alcohol. After centrifugation (13,000 rpm for 10 min) the released dye in supernatant was measured at 590 nm. One unit of the enzyme activity was defined as the amount of enzyme that liberated 1 µMol of mannose per min. Calculations were based on the standard curve supplied by Megazyme.

2.5.10 L-glutamic acid quantification

L-glutamic acid was quantified using a fluorescence-based assay, Amplex Red Glutamic acid/Glutamate oxidase assay kit (Invitrogen, UK). The assay was carried out according to the manufacturer's protocol. Fluorescence readings were made using a plate reader (FLUOstar Optima, BGM, UK) Resorufin, the Amplex Red reagent product was detected using an excitation and emission filters of 530 nm and 590 nm, respectively.

L-glutamic acid standard curve was constructed by serial dilution of L-glutamic acid stock solution (supplied in the kit) for the final concentration range of 0-10 µM. Diluted fermentation samples (50 µl) and control samples (positive:

20 mM H_2O_2 and negative: sample containing no L-glutamic acid) were dispensed into separate wells of a 96-well plate (Opaque, Cornwell).

Fifty microliters of Amplex® Red reagent/HRP/glutamate oxidase/glutamate-pyruvate transaminase/alanine working solution was added to each well. Incubation was carried out for 30 min at 37 ^{o}C in dark before the reading was taken. Background fluorescence correction was carried out by subtracting the negative control value (containing no L-glutamic acid) from the values obtained.

2.6 Calcium channel blocker studies

Elicitors (100 to 300 mg L^{-1}) and verapamil hydrochloride (100 µM) solutions were prepared and aseptically added to the production culture. Bacitracin A levels were measured as described before (Section 2.5.2). Experiments were carried out in triplicate.

2.6.1 Measurement of intracellular calcium levels

Measurement of intracellular Ca^{2+} levels was conducted as described before (Nair, 2007) with the following modifications: Whole cell extracts from cells harvested at the end of exponential growth phase were used for the intracellular Ca^{+2} studies. In order to determine the mechanism of the Ca^{2+} signalling, cell pellets were supplemented with: A) 1 mM Ca^{2+} and B) a mixture of 1 mM Ca^{2+} and 0.5 mM EDTA prepared in HPLC grade water. The cells were then incubated with 5 µM of Fluo-4 AM (Invitrogen) and 5 µM Calcein red-orange-AM (Invitrogen) for 30 min (37 ^{o}C) (in the dark). Thereafter samples were loaded in a 12 well micro plate for analysis. Hundred microliters of the elicitor, or 1 µM Ionomycin (positive control) prepared in DMSO, or 100 µL water (control cells) were added to the cells and the fluorescent measured (FLUOstar Optima (BGM) system) (1 per 1.46 sec) for 100 sec. The excitation filter was 485 nm and emission filter 520 nm. Control experiment was performed using water and ionomycin (positive control). No significant difference ($p \geq 0.05$) was observed in the wells with water addition. The fluorescence from each well within the plate was read and the data exported to Microsoft Excel for analysis.

2.7 Proteome studies

2.7.1 Protein extraction

Twenty millilitres of the cell suspension cultures was harvested by centrifugation at 5,000 rpm (Bio fresco, Heraeus) for 30 min at 4 ^{o}C. The cells were added to each FastPrep blue tube containing the lysing matrix and lysing buffer (0.5 mL of ice-cold buffer containing 20 mM Tris pH 8, 50 mg mL^{-1} DNAse, 50 mg mL^{-1} RNAse, 2.5 mM Pefabloc protease inhibitors, (Boehringer, Germany) and phosphatase inhibitors (100 mM of sodium orthovanadate and 50 mM of sodium fluoride). The mixture were shaken in the Fastprep instrument at a speed rating of 6.5 ms^{-1} for 45 s. Beads and cell debris were removed by centrifugation (13, 000 rpm, 15 min, 4 ^{o}C). The supernatant was then delipidated as described by Pro-Q Diamond manufacturer instructions. Six hundred microliters of methanol was added to the pellet together with 150 µL of chloroform and 450 µL of ultrapure water. The mixture was then mixed by vortexing, followed by centrifugation at 12, 000 rpm for 5 min (4 ^{o}C). The upper phase was then discarded and the white precipitation disc that forms between the upper and lower phase was kept. Finally 450 µL of methanol was added to the white precipitate followed by a centrifugation at 12, 000 rpm for 5 min (4 ^{o}C). The supernatant was discarded and the pellet dried for 5 min. The pellet was then re-dissolved in 200 µL rehydration buffer (6 M urea, 2 M thiourea, 0.5 % CHAPS (w/v) and 0.5 % (v/v) Pharmalyte (pH 3-10 or 4-7; Amersham) excluding Dithiothreitol (DTT). The extracts were stored in frozen aliquots at -80 ^{o}C.

2.7.2 Protein quantification

The protein content of each extract was performed according to the method described by Bradford, (1976). Ten microliters of each sample was diluted by making it up to 200 µL (in the same buffer) and mixed with 800 µL of Bradford reagent (Sigma). Absorbance was read at 595 nm in Novaspec II spectrophotometer (Amersham, UK). Standard curves were prepared using Bovine serum albumin (BSA) as standard over a concentration range of 0.1 to 1.5 mg mL^{-1} (R^2 = 0.9969 - see appendix 3).

2.7.3 Rehydration of IPG strip

Volumes containing 200 µg of *B. licheniformis* protein were made-up to 130 µL with rehydration buffer (6 M urea, 2 M thiourea, 0.5 % (w/v) CHAPS and 0.5 % (v/v) Pharmalyte (pH 3-10; Amersham) and 0.4 % (w/v) DTT). The mixture was loaded onto a swelling tray (Amersham, UK) and allowed to absorb onto a 7 cm Immobilised gel strip (IPG), pH 3-10/4-7 linear (GE healthcare) overnight. The strip was covered with mineral oil (Sigma-Aldrich, UK) during overnight rehydration. All experiments were carried out in replicates of 6, using protein samples from different cultures/extraction batches.

2.7.4 Isoelectric focusing

Following rehydration and protein absorption on gel strip, strips were gently blotted dry on tissue paper. Isoelectric focusing was carried out on a Multiphor II (Pharmacia BIOTECH) electrophoresis unit. Temperatures during isoelectric focusing were maintained at 17 °C using Amersham Multiptemp III thermostatic circulator cooling unit. Strips were placed gel-side-up onto the dry strip aligner tray and electrode strips were placed on the anode and cathode ends of the IPG strip after soaking them in distilled water. A small amount of mineral oil was poured onto the cooling ceramic block and the aligner tray was placed over the block. Electrodes were connected to the IEF electrophoresis unit and the IPG strips were focused for 4 hrs and 15 min, using an Amersham EPS 3501power pack with the following voltage profile: a linear increase from 0 to 300 V over 30 min, a linear increase from 300 to 600 V over 30 min then a linear increase from 600 to 3500 V over 3 hrs and 5 min.

2.7.5 Equilibration of IPG strips

After isoelectric focusing, strips were equilibrated to reduce disulfide bonds and unfolding of proteins with a view to promoting the transfer of proteins from the IPG strips onto SDS-PAGE. Equilibration was performed for 15 min by rocking the strips gently in 5 mL of 50 mM Tris-HCl, pH 8.8; 6 M urea; 30 % (v/v) glycerol; 2 % (w/v) sodium dodecyl sulphate (SDS); and 1 % (w/v) DTT and, for

another 15 min in 5 mL of 50 mM Tris-HCl, pH 8.8; 30 % (v/v) glycerol; 2 % (w/v) SDS; and 2.5 % (w/v) iodoacetamide.

2.7.6 SDS-PAGE

The second dimension was run by placing the focused and equilibrated strips onto a 1 mm thick 8 x 7 cm 12 % (w/v) SDS-PAGE resolving gel. SDS-PAGE resolving gels were prepared by mixing water (4.9 mL), 1.5 M Tris base (pH 8.8; 2.6 mL), 40 % Bis acrylamide (3 mL), 10 % (w/v) ammonium persulfate (100 μL), N,N,N',N'-tetramethylethylenediamine (TEMED) (10 μL), and 10 % (w/v) SDS (100 μL) and allowed to set within the gel plates. Peppermint Stick^{TM} phosphoprotein molecular weight marker (Invitrogen) was loaded onto a small piece of filter paper placed on the low pH end of the strip. IPG strips were sealed onto the SDS-PAGE resolving gel by pouring sealing buffer (0.5 % (w/v) agarose, 25 mM Tris, 192 mM glycine, 0.1 % SDS and trace amounts of bromophenol blue over the strips. This ensured that the strip was intact with the resolving gel during electrophoresis (protein separation).

Electrophoresis was carried out using a Biorad protein electrophoresis unit at 150 V in Laemmli electrolyte buffer (1.92 M Glycine, 0.25 M Tris Base, and 1 % SDS (w/v)). Altogether, 20 gels were run with different biological samples for each of test and control cultures, out of which 5 gels (most uniform) were used subsequently for image analysis.

2.7.7 Protein visualisation and Image analysis

After migration of the proteins in 2-DE, the gels were stored in 2 % (v/v) acetic acid at 4 ^{o}C. Gels were stained by incubating four times for 20 min in 100 mL of water then stained by shaking in Pro-Q Diamond stain (Invitrogen). The Pro-Q Diamond stain was used according to the manufacturer's instructions. After staining, gels were shaken twice for 10 min (each time) in water before imaging, using a Typhoon (Perkin Elmer), with excitation and emission filters of 560-580 nm. After imaging to detect the phosphoproteins, gels were stained for all the proteins with colloidal Coomassie Brilliant blue G-250. Stained gels were scanned using a GS800 densitometer (Bio-Rad, U.K.) and the resulting images

were acquired with Quantity II software. Each image was converted to a TIFF file and analysed using Progenesis PG240 Samespot software (Nonlinear Dynamics, UK). Five gels from each of test and control batches were uploaded onto the Progenesis PG 240 Samespot software for analysis. The amount of a protein in a particular spot was calculated based on the volume of that spot. To reflect the quantitative variations in the intensity of proteins spots between gel images of control and elicited cultures, the spot volume was normalised as a percentage of the total volume of all protein spots on the corresponding gel image. Spots were ranked by *p-value* for one way ANOVA analysis and fold changes were calculated using the spot normalised volume. *p-values* ≤ 0.05 were considered significant.

2.7.8 Sequencing (LC-MS/MS) and identification

Spots, which differed significantly in volume between control and test cultures, were excised and dispatched to the University of York Mass Spectrometry Facility (UK) where they were commercially sequenced by LC-MS/MS and identified by sequence matching on the NCBI database. Figure 2.2 summarises the proteomics procedure.

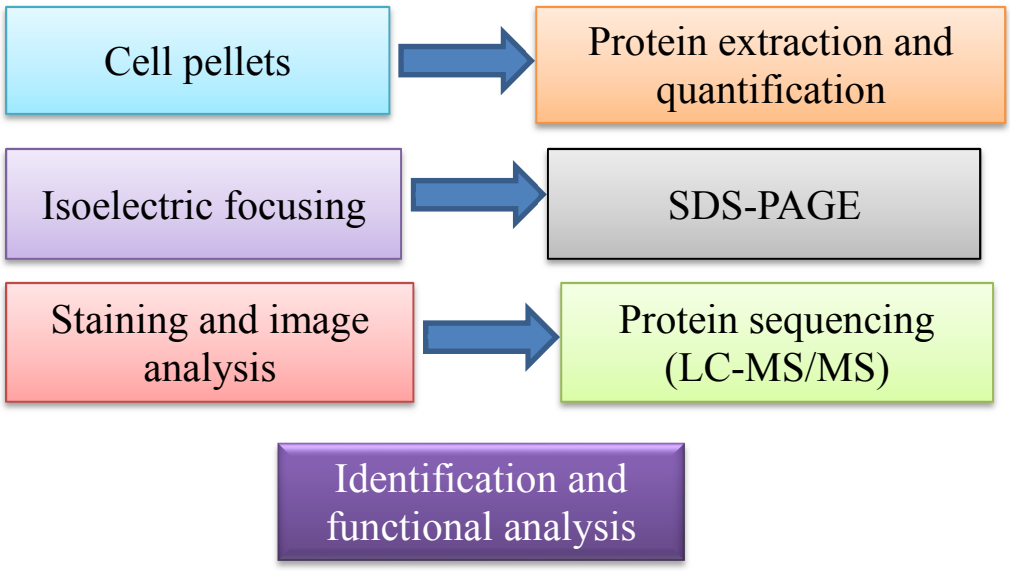


Figure 2.2 Flow diagram showing the functional proteomic strategy used in the study.

2.8 Drawing software and Data analysis

All graphs were produced using Microsoft Excel (Microsoft Corporation) and Error bars indicate standard deviation between triplicate samples. Paired t-test was used for performing comparisons between the control and test conditions. $p \leqslant 0.05$ (∗), p , 05 (∗∗) and p and (∗∗∗) were considered significant.

Chapter 3

3.0 Results

3.0 Elicitation studies in *Bacillus licheniformis*

This chapter presents the physiological and metabolic responses caused by the oligosaccharide elicitors (OM, OG and MO) in cultures of *B. licheniformis*. Studies were conducted in shaken flasks (500 mL) and stirred tank reactors (0.6-L and 2.5-L), in the presence (test cultures) and absence (control cultures) of elicitors. All the samples taken from these cultures were assayed for bacitracin A concentration, growth and pH. TLC and HPAEC analyses were carried out to investigate the fate of MO during the growth of the bacterium. Analysis of β-mannanase activity was also performed. These results are presented in Sections 3.1 to 3.4 of this thesis.

Changes at a molecular level caused by the elicitors are shown in Sections 3.5 and 3.6. A comparative proteome and phosphoproteome analysis were carried out on intracellular proteins extracted from a 24 hrs and 48 hrs old cultures of the *B. licheniformis*.

Effect of the oligosaccharide elicitors on ROS, SOD and catalase is also reported in Section 3.6.

3.1 Shake flask fermentations

Previous elicitation studies in *B. licheniformis* cultures exploring optimal elicitation conditions for the overproduction of bacitracin A, have shown that addition of OG: 100 mg L^{-1}, OM: 200 mg L^{-1} and MO: 300 mg L^{-1} at 24 hrs, results in higher bacitracin A production (Murphy, 2008). However, there are no reports on the effect of lower or higher concentrations of these oligosaccharides on the production of bacitracin A.

The effect of lower and higher concentrations (25 to 1000 mg L^{-1} - Table 2.2) of these elicitors on bacitracin A production was investigated. Optimum concentrations (OG: 100 mg L^{-1}; OM: 200 mg L^{-1}; and MO: 300 mg L^{-1}), were also studied for comparison and are presented below.

3.1.1 Effect of optimum concentrations of elicitors on cell growth and bacitracin A production

Cell growth and pH profiles of *B. licheniformis* are shown in Figure 3.1. Optimum concentrations (100 – 300 mg L^{-1}) of OG, OM and MO (as suggested by Murphy *et al*., 2007a) caused no significant elicitation difference ($p \geq 0.05$) in pH and cell growth profiles between the control and the treatments (Figure 3.1).

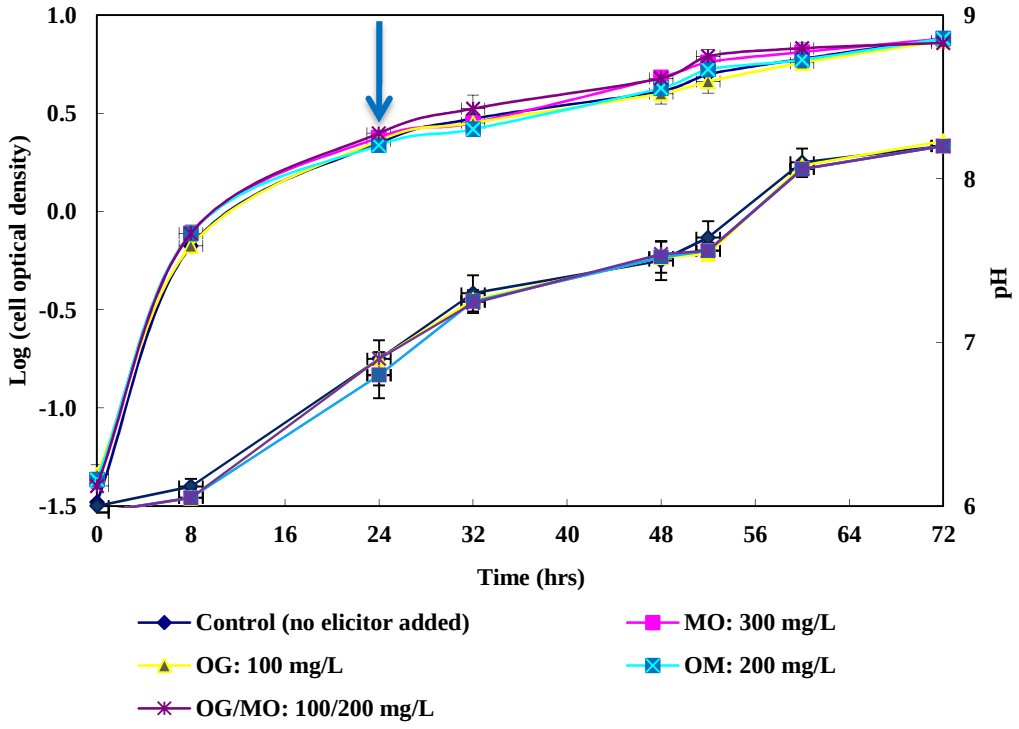


Figure 3.1 Cell growth and pH profile of *B. licheniformis*. Control (no elicitor), MO: 300 mg L^{-1}; OG: 100 mg L^{-1}; OM: 200 mg L^{-1}; OG: 100 mg L^{-1} and MO 200: mg L^{-1}. The arrow represents the time of addition of the elicitors to the culture. Error bars indicate standard deviation between triplicate samples.

Figure 3.2 shows the effect of the optimum concentrations (100 – 300 mg L^{-1}) of OG, OM and MO additions at 24 hrs on bacitracin A concentration. At 48 hrs, culture supplementation with OG (100 mg L^{-1}) resulted in the highest bacitracin A increase (36 %), followed by OG/MO: 100/200 mg L^{-1}, (32 %) and MO: 300 mg L^{-1}, (30 %).

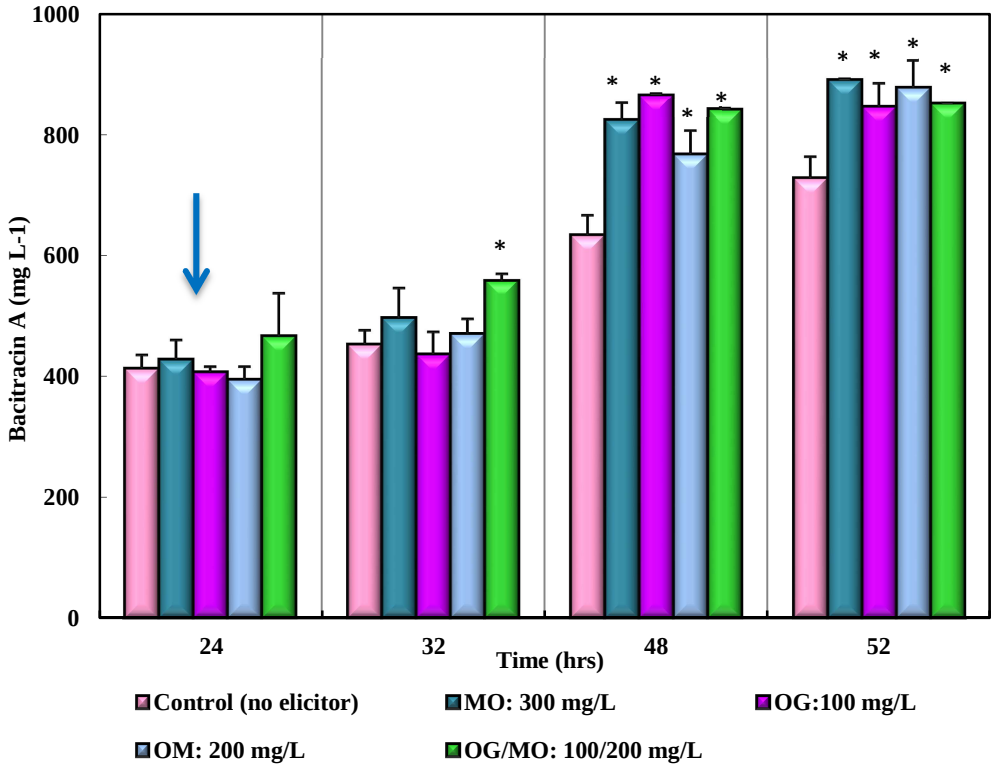


Figure 3.2 Effect of elicitors on bacitracin A production in cultures of *B. licheniformis* under optimal conditions: Control: (no elicitor), MO: (300 mg L^{-1}); OG: (100 mg L^{-1}); OM: (200 mg L^{-1}); OG: (100 mg L^{-1}) and MO: (200 mg L^{-1}). The arrow represents the time of addition of the elicitors to the culture. Error bars indicate standard deviation between triplicate samples.

3.1.2 Effect of lower concentrations of elicitors on cell growth and bacitracin A production

Cell growth and pH profiles of *B. licheniformis* are shown in Figure 3.3. Lower concentrations (25 – 50 mg L^{-1}) of OG, OM and MO, caused no significant elicitation difference ($p \geq 0.05$) in pH and cell growth profiles between the control and the treatments.

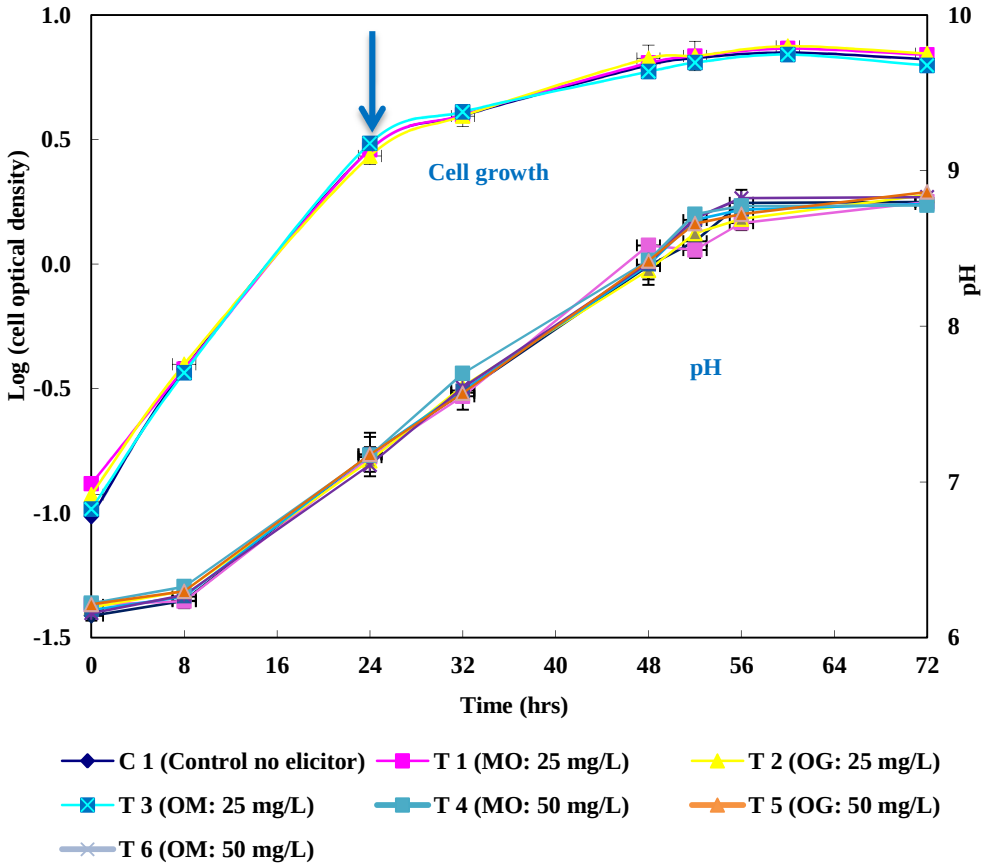


Figure 3.3 Effect of lower concentrations (25 – 50 mg L^{-1}) of MO, OM and OG on pH and cell growth of *B. licheniformis* as compared to control culture (C 1). The arrow represents the time of addition of the elicitors to the culture. Error bars indicate standard deviation between triplicate samples.

The results shown in the Section 3.1.1, demonstrated that the addition of OG: 100 mg L^{-1} at 24 hrs after *B. licheniformis* inoculation resulted in higher antibiotic yields with no significant change in biomass production. Similar results were observed elsewhere (Murphy, 2008). In this investigation concentrations of elicitors lower than 100 mg L^{-1} added at 24 hrs did not have any significant ($p \geq 0.05$) effect on the bacitracin A (Figure 3.4).

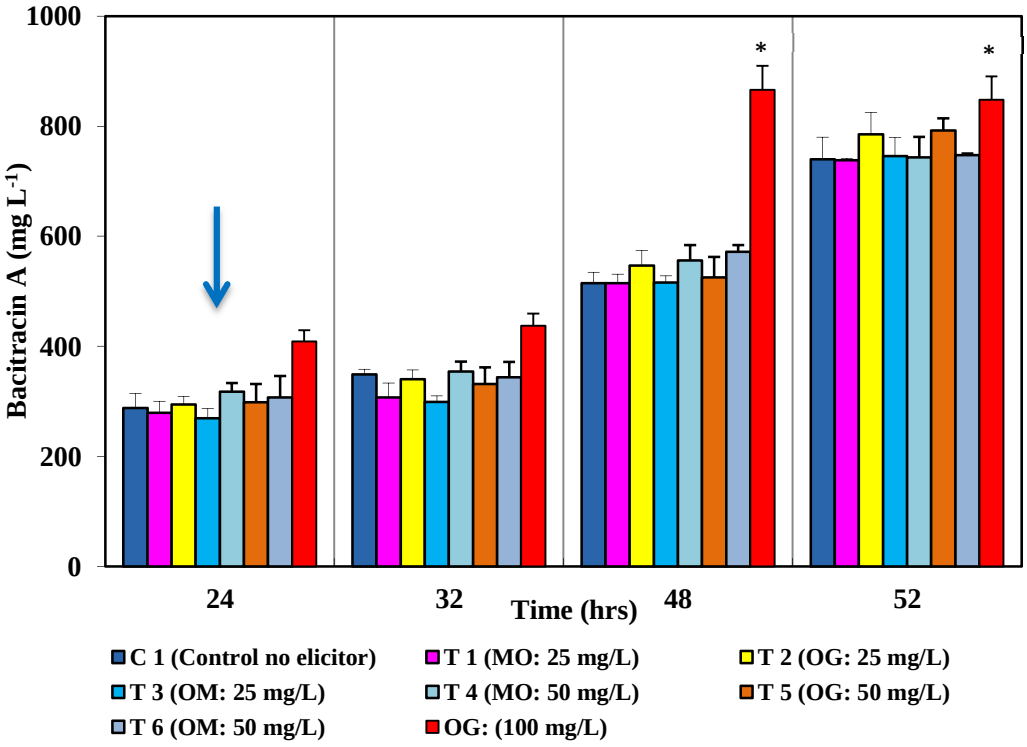


Figure 3.4 Bacitracin A production profile in *B. licheniformis* culture elicited with lower concentrations (25 – 50 mg L^{-1}) of MO, OM and OG elicitors. Control culture (C 1: no elicitor added), OG: 100 mg L^{-1} was used as positive control. The arrow represents the time of addition of the elicitors to the culture. Time points represent the samples taken during the fermentation growth. Error bars indicate standard deviation between triplicate samples.

3.1.3 Effect of higher concentrations of elicitors on cell growth and bacitracin A production

Biomass profiles shown to be significantly different ($p \leq 0.05$) when cells were treated with higher concentrations (500 – 1000 mg L^{-1}) of the elicitors: OG (1000 mg L^{-1}) addition resulted in the highest biomass increase (63 %), followed by OM at 500 mg L^{-1} (52 %); OM at 1000 mg L^{-1} (51 %) and MO at 1000 mg L^{-1} (48 %) at 56 hrs, as observed in Figures 3.5 and 3.6. No significant difference ($p \geq 0.05$) was observed in pH profile between control and test cultures.

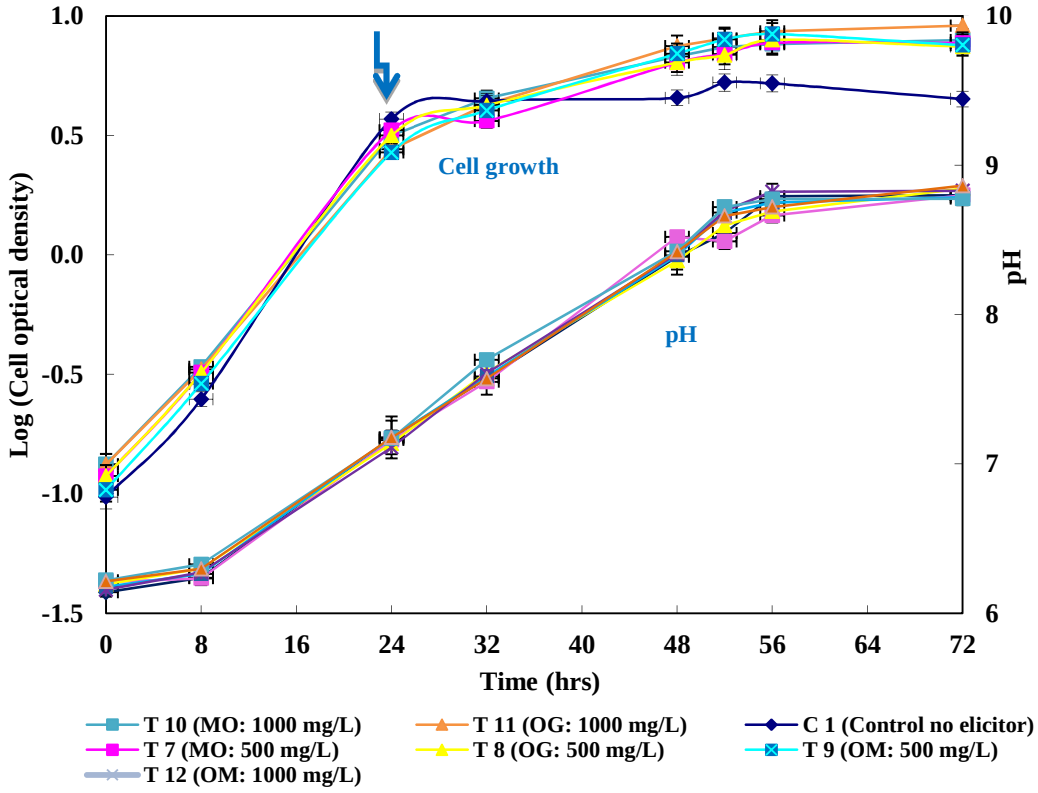


Figure 3.5 Effect of higher concentrations (500 – 1000 mg L^{-1}) of MO, OM and OG on pH and cell growth of *B. licheniformis* as compared to control culture (C 1). The arrow represents time of addition of the elicitors to the culture. Error bars indicate standard deviation between triplicate samples.

Figure 3.6 shows biomass increase (%) caused by OG, OM and MO at higher concentrations (500 – 1000 mg L^{-1}) as compared to the control cells. Significant difference between control and tests was clearly observed from 48 hrs onwards.

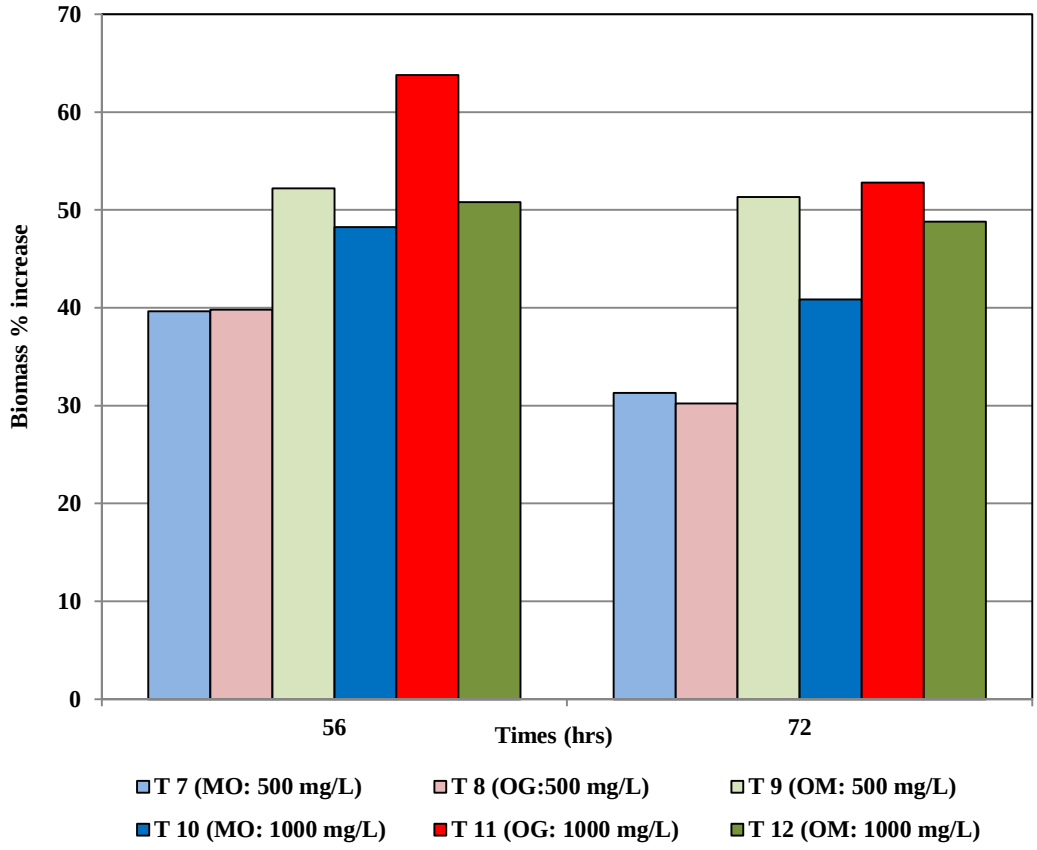


Figure 3.6 Biomass increase of *B. licheniformis* as response of the addition of higher concentrations (500 – 1000 mg L^{-1}) of MO, OM and OG elicitors at 24 hrs, as compared to control culture. Time points represent the samples taken at 56 and 72 hrs after inoculation.

Figure 3.7 shows the effect of OG, OM and MO additions at 24 hrs on bacitracin A concentration. At 52 hrs, culture supplementation with MO: (500 mg L^{-1}) and OG: (1000 mg L^{-1}) resulted in the highest bacitracin A increase (23 %) each, followed by OM: 500 mg L^{-1}, (20 %). However, as presented before, the increase was concomitant with enhancement in biomass concentration (see Figure 3.6).

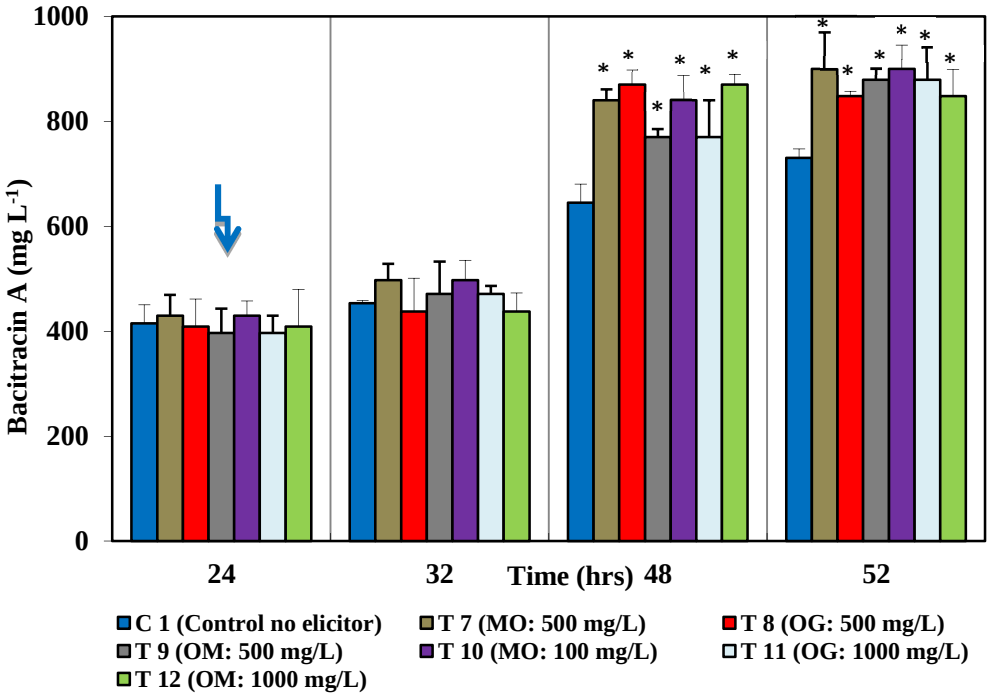


Figure 3.7 Bacitracin A production profile in *B. licheniformis* culture elicited with higher concentration (500 – 1000 mg L^{-1}) of MO, OM and OG elicitors. Control culture (C 1). The arrow represents time of addition of the elicitors to the culture. Error bars indicate standard deviation between triplicate samples.

3.2 Fate of Mannan oligosaccharides in cultures of *B. licheniformis*

3.2.1 MO composition determination

M20 medium used for the growth did not contain any carbohydrate as a carbon source (L-glutamic acid was used instead). This facilitated the opportunity to follow the fate of the elicitors during the fermentation by utilising phenol sulphuric acid assays. Nevertheless, it was found that precise detection was masked due the production of carbohydrates by the microorganism (Figure 3.8). For this reason a specific method for its detection was essential.

Figure 3.8 shows the carbohydrate profile at different time points during the shaken flask fermentation between the control and the test culture (MO: 300 mg L^{-1} added at 24 hrs). Carbohydrate concentration was significantly higher in the test (due the addition of MO). Control culture showed a slow and gradual increase in carbohydrate concentration (due to the production of exopolysaccharides). A similar profile was presented elsewhere (Murphy, 2008).

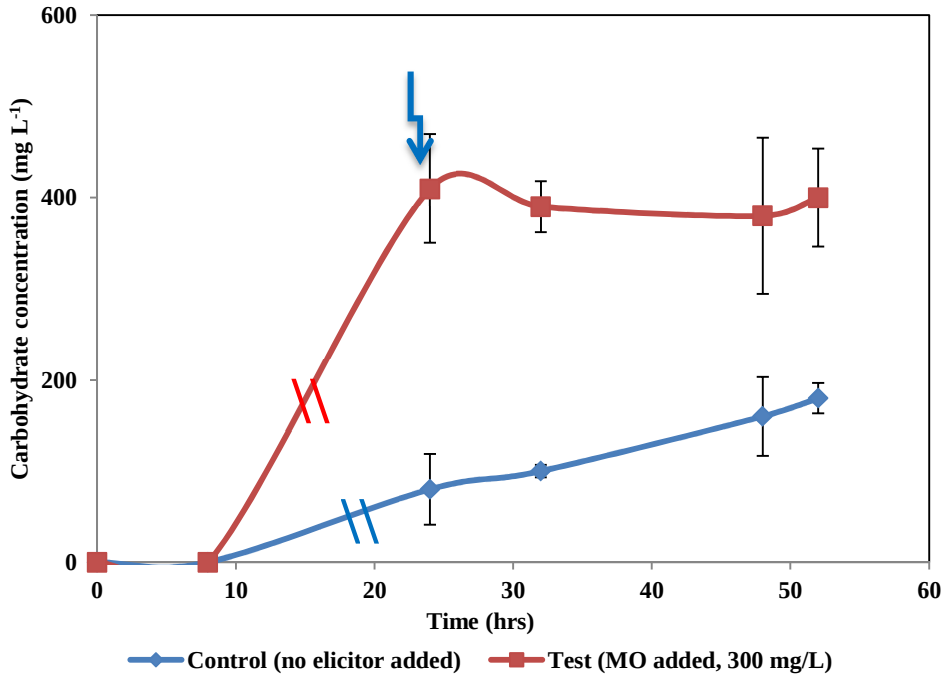


Figure 3.8 Carbohydrate profile in *B. licheniformis* culture in the presence of MO (300 mg L^{-1}). Control culture (no elicitor added). Experiments were carried out in triplicates. Error bars indicate standard deviation between triplicate samples. Arrow represents the addition of MO at 24 hrs.

This study used TLC (thin layer chromatography) and HPAEC (high performance anion exchange chromatography) for MO characterisation and detection. Figure 3.9 shows the separation profile of the soluble fraction of MO on TLC (3.9 A) plate and (3.9 B) HPAEC, respectively. The first component to elute was mannose (retention time 1.40 min) and the last mannooctaose (retention time 11.23 min). The first dark and strong dot observed on the TLC plate suggests that MO sample had an insoluble part. Further investigation is required to reveal the composition of this insoluble fraction.

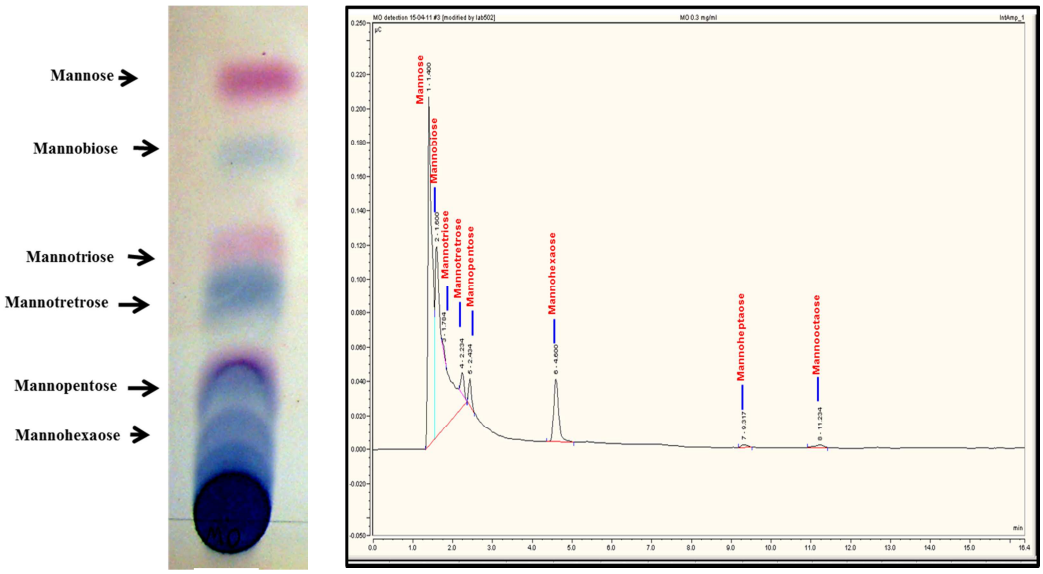


Figure 3.9 MO profile by (A) TLC and (B) HPAEC.

Maltodextrin was used as standard for further confirmation of DP (degree of polymerisation) of the peaks (Figure 3.10).

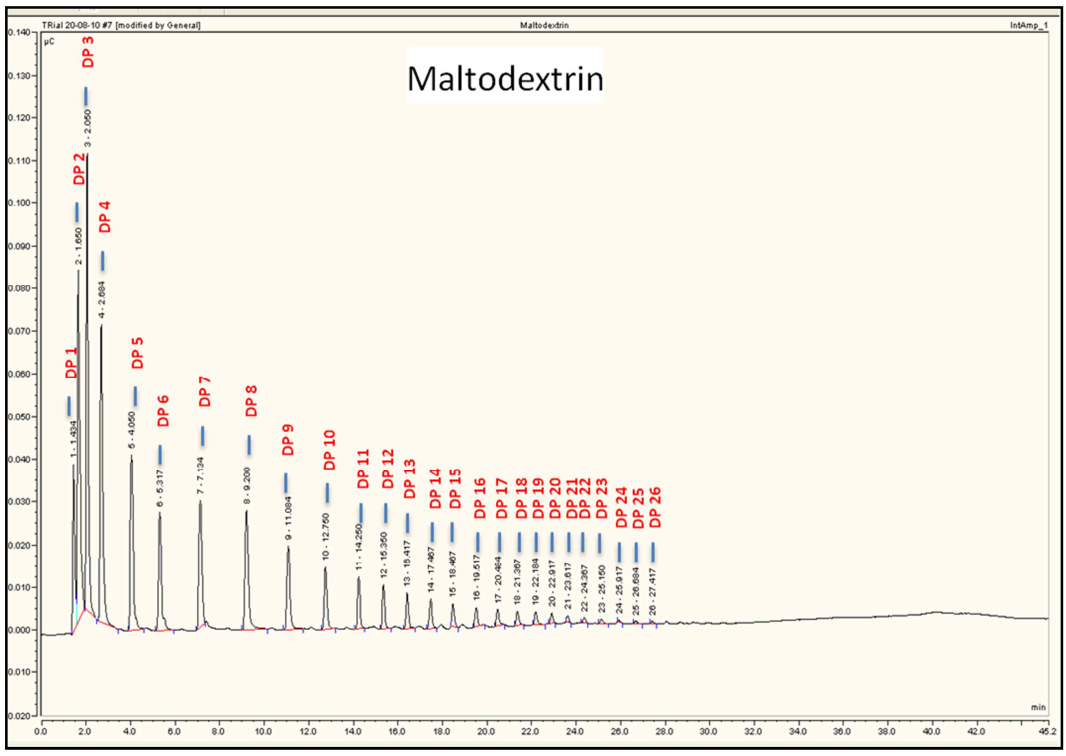


Figure 3.10 Maltodextrin (525 µg mL^{-1}) spectrum by HPAEC. (DP: degree of polymerisation).

In order to calculate the concentration of the MO constituents, a standard curve was prepared using MO as standard over a concentration range of 0 to 3 mg mL^{-1}. The peaks of the constituents were identified using commercially pure standards (mannose, mannobiose, mannotriose, mannotetrose, mannoheptaose) and their concentrations were calculated. Mannobiose (45.4 %) and Mannose (40.4 %) were shown to be the main MO components (Figure 3.11).

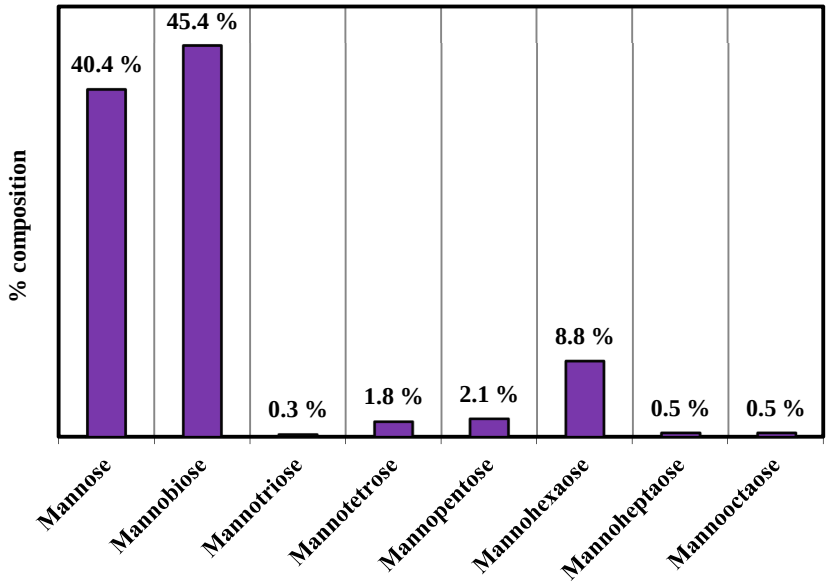


Figure 3.11 Mannan oligosaccharides (300 mg L^{-1}) composition.

3.2.2 Detection of MO during the fermentation process

In this study MO (300 mg L^{-1}) was added to *B. licheniformis* culture at 24 hrs and the fermentation was carried out for 72 hrs. Samples were taken through the course of the fermentation and centrifuged. The supernatant was freeze dried and re-dissolved into 100 µl of distilled water and tested for the presence of MO using TLC and HPAEC methods.

Figure 3.12 shows the presence of MO in the supernatant of the samples taken throughout the *B. licheniformis* growth. TLC results revealed that only some constituents of MO could be spotted after its addition to the culture at 24 hrs (Lane 4). These constituents then disappeared with time (Lanes: 6, 8 and 10). No spots were observed in control cultures (Lanes: 3, 5, 7 and 9). M20 medium was also run on TLC plates; no spots were observed (appendix 4).

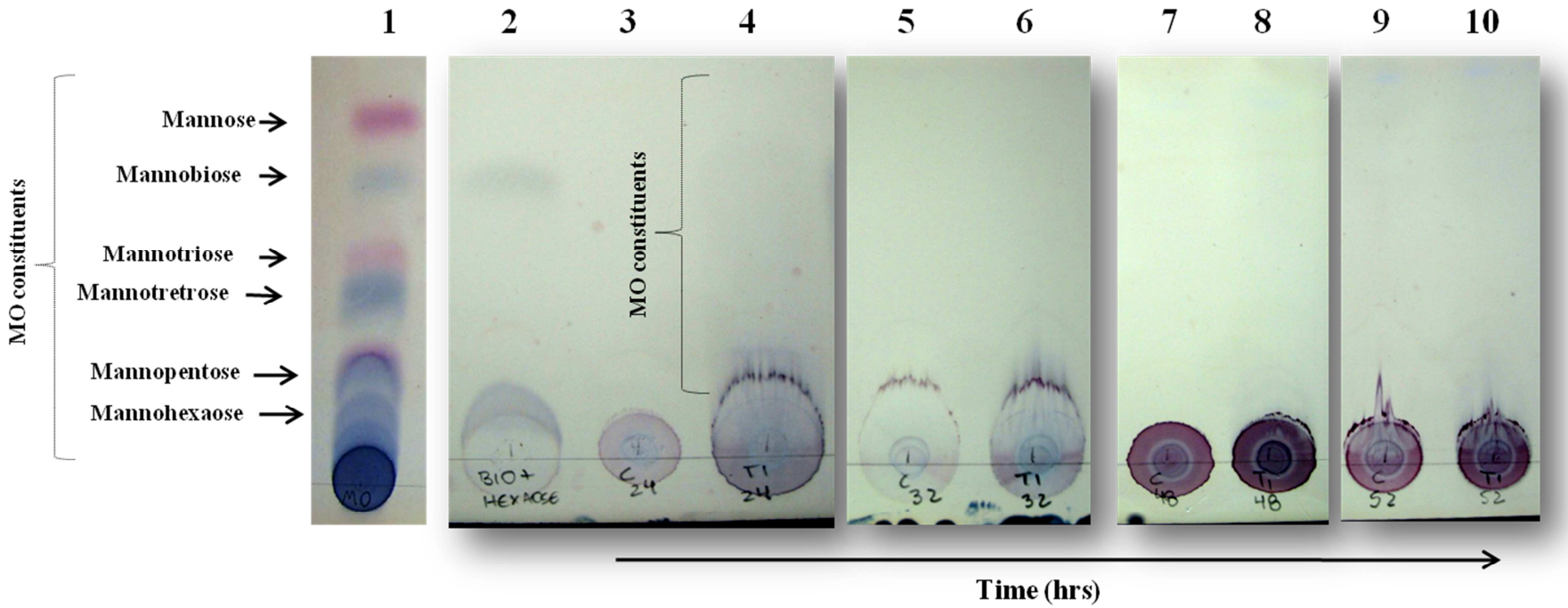


Figure 3.12 TLC profile of MO during the course of the fermentation of *B. licheniformis*. Lanes: 1. Standard MO; 2. Standard Mannobiose + Mannohexaose; 3. Control 24 hrs (no elicitor added); 4. Test 24 hrs (MO: 300 mg L^{-1}); 5. Control 32 hrs (no elicitor added); 6. Test 32 hrs (MO: 300 mg L^{-1}); 7. Control 48 hrs (no elicitor added); 8. Test 48 hrs (MO: 300 mg L^{-1}); 9. Control 52 hrs (no elicitor added); 10. Test 24 hrs (MO: 300 mg L^{-1}). Experiments were carried out in triplicates.

More accurate quantitative results were obtained from HPAEC analysis. The concentration of the residues is presented in Figure 3.13 which shows the MO concentration at different stages of the shaken flask fermentation. Lane 1, represents the main MO components (mg mL^{-1}) added to the cultures of *B. licheniformis*. As soon as MO was added to the culture broth, a drastic decrease in MO concentration was observed (Lane 2). Mannose and mannobiose concentrations decreased from 0.121 and 0.136 mg mL^{-1} to approximately 0.016 and 0.014 mg mL^{-1} respectively. Mannohexaose could not be detected in samples taken after 32 and 48 hrs, respectively. Mannose was the last component to be detected at 48 hrs. No MO component was observed after this time point. This profile suggested that MO was broken down and possibly utilised by the microorganism over the course of fermentation.

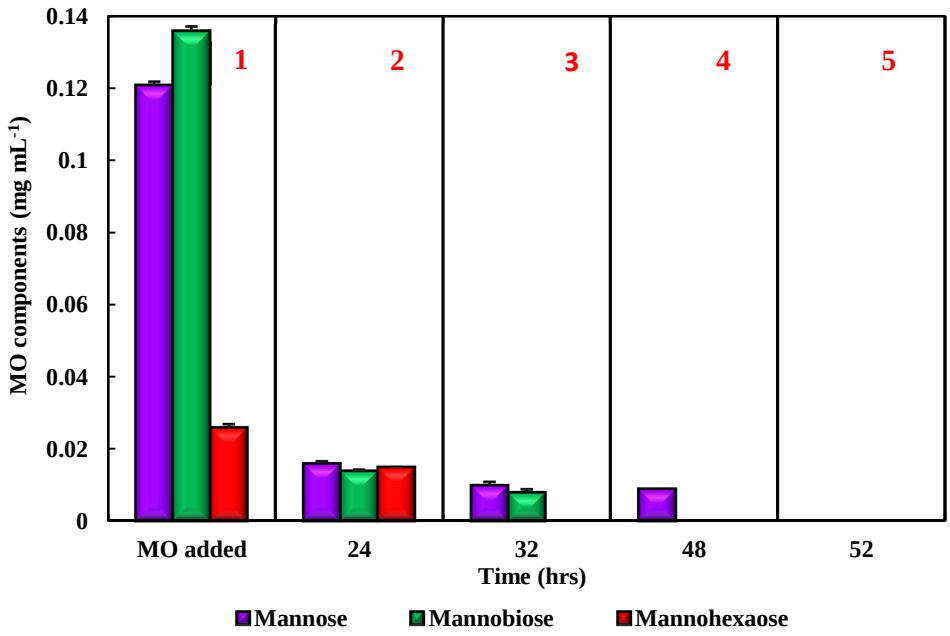


Figure 3.13 Degradation/utilisation of MO during the course of fermentation of *B. licheniformis*. 1= MO main components without the presence of *B.licheniformis* cells; 2 = MO in the presence of *B. licheniformis* at 24 hrs*;* 3= MO profile after 32 hrs; 4= MO profile after 48 hrs; 5 = MO profile after 52 hrs. Error bars indicate standard deviation between triplicate samples.

3.2.3 β-mannanase activity

The results presented in Section 3.2.2, suggest enzymatic breakdown of MO during the *B. licheniformis* growth. ß-mannanase is an inducible enzyme that catalyses hydrolysis of substrates such as mannan. Through a multi-step reaction, β-mannanase degrades mannan residues to the end product, mannose (Dhawan and Kaur, 2007). As an analogous pattern was observed (Figure 3.14) where the end product detected was mannose (Lane 4) the β-mannanase activity assay was carried out on samples taken through the course of *B. licheniformis* growth. The culture was supplemented with MO (300 mg L^{-1} added at 24 hrs); no elicitor was added to the control culture. Figure 3.12 shows β−mannanase activity in cultures of *B. licheniformis*. It was observed that β−mannanase was induced as soon as MO was added into the culture and its activity was steadily increased during the fermentation. Maximum activity of approximately 26.8 U mL^{-1} was observed in cultures supplemented with MO. ß−mannanase was also produced by control cultures at a later stage (after 52 hrs), reaching a maximum activity of 18.1 U mL^{-1}, suggesting that the carbohydrates produced by the control cells induce the enzyme production, possibly via the carbohydrate sensory system or lectin type receptor. Nevertheless, the activity of the test culture was still significantly higher (64 %) ($p \leq 0.05$) than the control culture at 52 hrs.

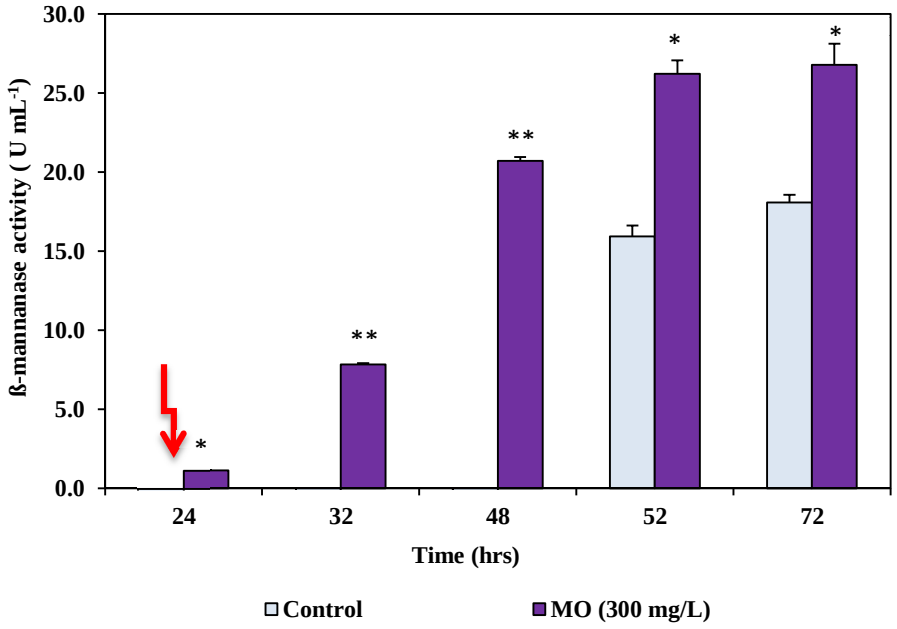


Figure 3.14 β-mannanase activity during the course of fermentation of *B. licheniformis*. Control (no elicitor added) and Test (300 mg L^{-1} added at 24 hrs – red arrow). Error bars indicate standard deviation between triplicate samples.

3.2.4 Relationship between β-mannanase activity and total carbohydrate concentration

Figure 3.15 shows the relationship between β-mannanase activity and total carbohydrate concentration. β-mannanase was induced as soon as MO was added into the culture and its activity was steadily increased during the fermentation. Production of carbohydrate was observed as early as 24 hrs after inoculation, in both control and test culture. In the control culture (no elicitors added) β-mannanase activity was just observed after 52 hrs (late stationary phase), suggesting that the carbohydrate likely to induce β-mannanase is not produced until a later stage of bacterial growth.

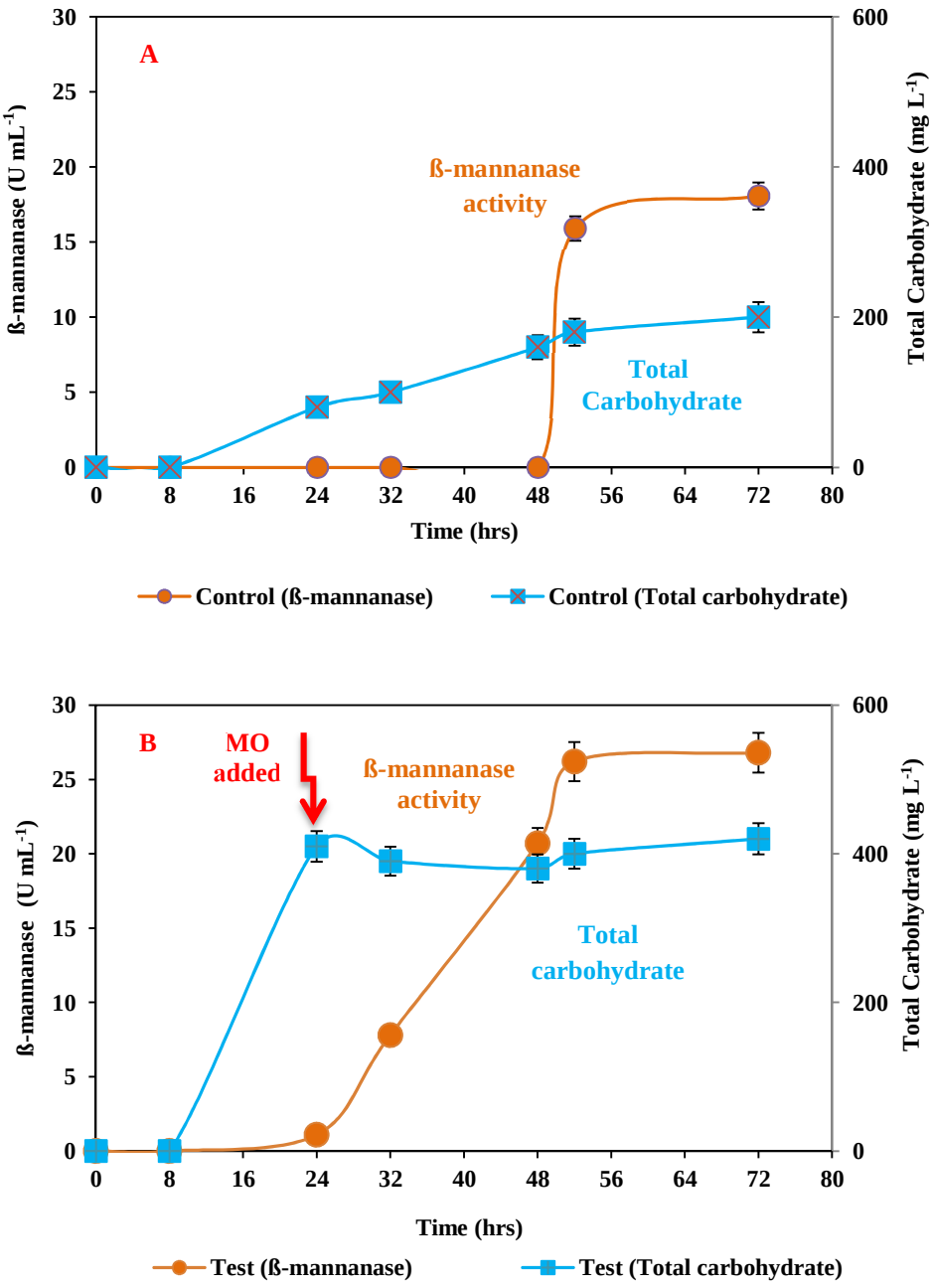


Figure 3.15 β-mannanase activity and total carbohydrate production during the course of fermentation of *B. licheniformis*. (A) Control (no elicitor added) and (B) Test (300 mg L^{-1} at 24 hrs). Experiments were carried out in triplicate. Error bars indicate standard deviation between triplicate samples.

3.3 Effect of the degradation products of MO (Mannose, Mannobiose, and Mannohexaose) on bacitracin A production

The major constituents of MO (mannose, mannobiose and mannohexaose) were individually added into the culture of *B. licheniformis* to investigate their potential role as elicitors in production of bacitracin A. The concentrations used were based on the earlier established MO composition; Mannose (121 mg L^{-1}), Mannobiose (136.2 mg L^{-1}) and Mannohexaose (26.5 mg L^{-1}) in 300 mg L^{-1} MO. MO-treated (300 mg L^{-1}) culture was performed as comparison.

3.3.1 Cell growth and pH profiles

There was no significant difference ($p \geq 0.05$) in the growth and pH profiles of the control (no elicitor added) and the elicited cultures (Figure 3.16).

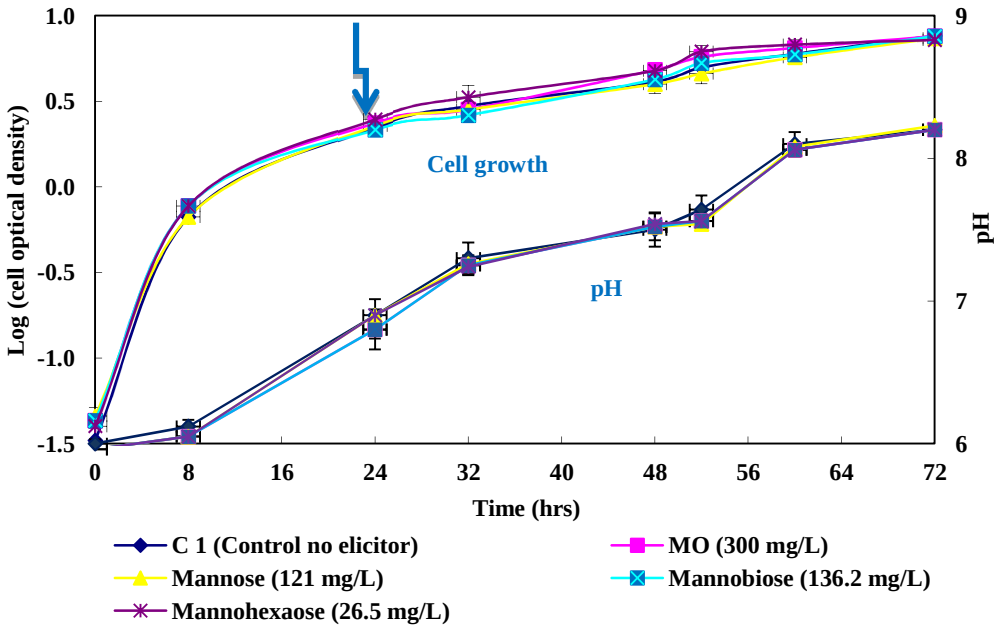


Figure 3.16 The effect of Mannose (121 mg L^{-1}), Mannobiose (136.2 mg L^{-1}) and Mannohexaose (26.5 mg L^{-1}) on pH and cell growth as compared to control culture (no elicitor added). Concentrations used were based on the constituents' concentrations of MO. The arrow represents the time of addition of the saccharides to the cultures. Error bars indicate standard deviation between triplicate samples.

3.3.2 Bacitracin A production

Figure 3.17 shows enhanced bacitracin A production in all the test cultures as compared to the control (no elicitors added). The addition of mannose and mannohexaose caused an increase of 10 % and 35 % on bacitracin A production at 32 hrs, respectively. The effect caused by mannose and mannohexaose was then quenched right after 48 hrs. Highest bacitracin A production levels of 891 mg L^{-1} and 860 mg L^{-1} were observed at 52 hrs in test cultures supplemented with MO and mannobiose ($p \leq 0.05$), respectively, as compared to 728 mg L^{-1} in control cultures. Interestingly, similar levels of bacitracin A were observed throughout the course of the fermentation in cultures treated with MO and mannobiose.

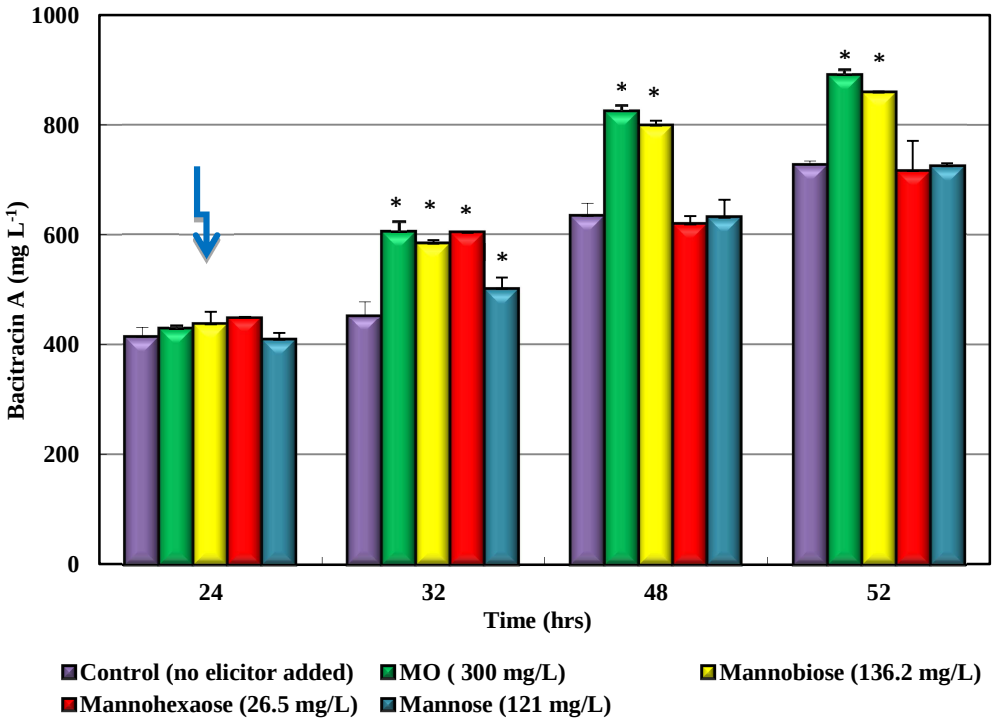


Figure 3.17 Effect of Mannose, Mannobiose and Mannohexaose on bacitracin A production in cultures of *B. licheniformis*. Control (no elicitor added), MO (300 mg L^{-1}); Mannose (121 mg L^{-1}); Mannobiose (136.2 mg L^{-1}) and Mannohexaose (26.5 mg L^{-1}). The arrow represents the time of addition of the saccharides to the cultures Concentrations used were based on the constituents' concentrations in MO. Error bars indicate standard deviation between triplicate samples.

3.3.3 HPAEC degradation profile of Mannose, Mannobiose and Mannohexaose in *B. licheniformis* culture

Figures 3.18, 3.19 and 3.20 show mannose, mannobiose and mannohexaose concentrations at different stages of the shaken flask fermentations. Profile 1 represents the initial oligosaccharide concentration (mg mL^{-1}) added to the culture. As they were added to the culture broth, profile 2, a dramatic decrease was observed in their concentrations in all the cases. Mannose is no longer detected after 48 hrs of post-inoculation whereas in mannobiose supplemented cultures, mannobiose was demonstrated to be decomposed into mannose residues.

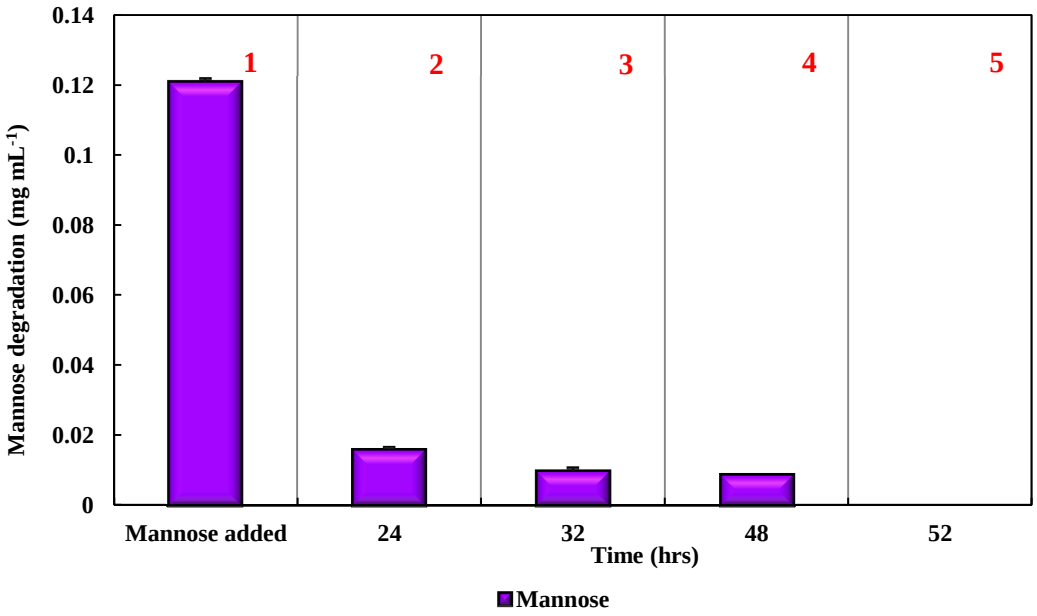


Figure 3.18 HPAEC degradation/utilisation profile of Mannose during the course of fermentation of *B. licheniformis*. 1= Mannose (120 mg L^{-1}) cell free; 2 = Mannose in the presence of *B. licheniformis* after 24 hrs; 3= Mannose profile after 32 hrs; 4= Mannose profile after 48 hrs; 5 = Mannose profile after 52 hrs. Experiments were carried out in triplicates. Error bars indicate standard deviation between triplicate samples.

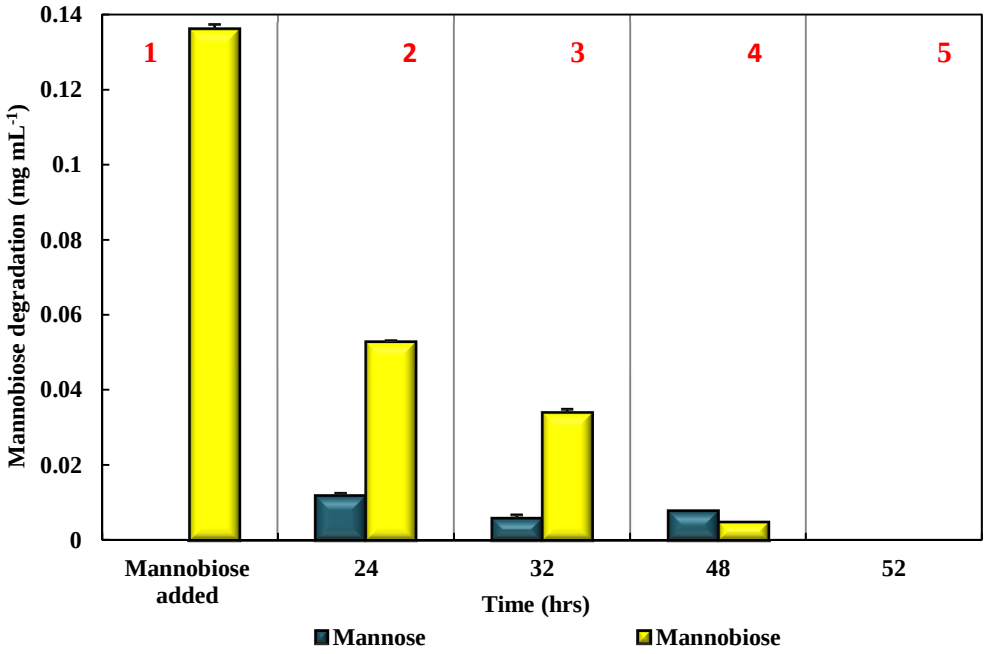


Figure 3.19 HPAEC degradation/utilisation profile of Mannobiose during the course of fermentation of *B. licheniformis*. 1= Mannobiose (136 mg L^{-1}) cell free; 2 = Mannobiose in the presence of *B. licheniformis* at 24 hrs; 3= Mannobiose profile after 32 hrs; 4= Mannobiose profile after 48 hrs; 5 = Mannobiose profile after 52 hrs. Experiments were carried out in triplicates. Error bars indicate standard deviation between triplicate samples.

Similar profile was seen in Mannohexaose treated culture. A degradation of mannohexaose into a mannose end residues was clearly observed (Figure 3.20).

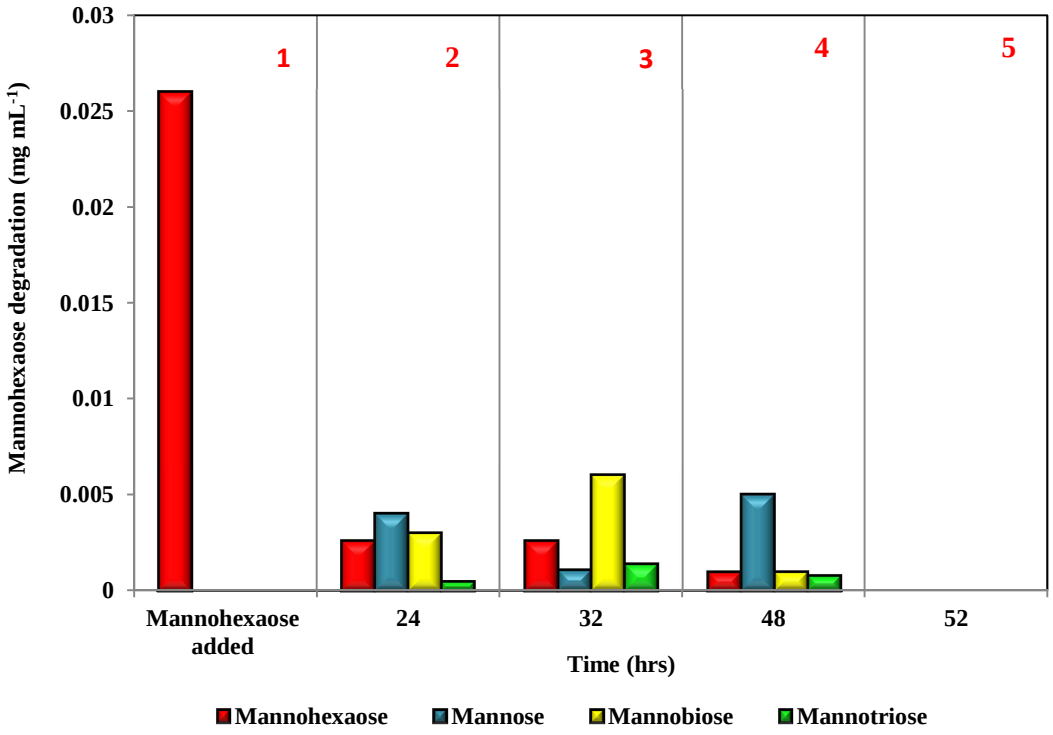


Figure 3.20 HPAEC degradation/utilisation profile of Mannohexaose during the course of fermentation of *B. licheniformis*. 1= Mannohexaose (0.26 mg L^{-1}) cell free; 2 = Mannohexaose in the presence of *B. licheniformis* at 24 hrs; 3= Mannohexaose profile after 32 hrs; 4= Mannohexaose profile after 48 hrs; 5 = Mannohexaose profile after 52 hrs. Experiments were carried out in triplicates. Error bars indicate standard deviation between triplicate samples.

3.3.3.1 β-mannanase activity

Figure 3.21 shows β-mannanase activity in cultures of *B. licheniformis* elicited with mannose, mannobiose and mannohexaose. ß-mannanase activity was detected in the presence of mannobiose and mannohexaose as soon as they were added to the cultures (at 24 hrs). β-mannanase activity was also produced by mannose and control cultures at a later stage (after 52 hrs). The highest β-mannanase activity of 19 U mL^{-1} and 18 U mL^{-1} was reached by mannobiose and control cultures after 52 and 72 hrs, respectively.

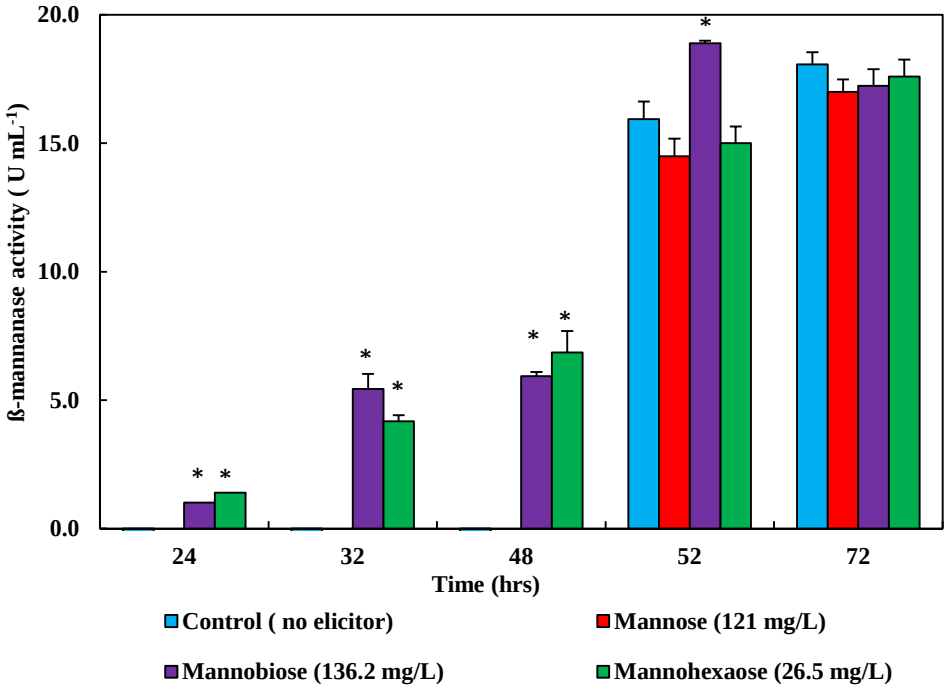


Figure 3.21 ß-mannanase activity during the course of fermentation of *B. licheniformis*. Control (no elicitor added), Mannose (121 mg L^{-1}), Mannobiose (136.2 mg L^{-1}) and Mannohexaose (26.5 mg L^{-1}) added at 24 hrs. Experiments were carried out in triplicates. Error bars indicate standard deviation between triplicate samples.

3.4 Elicitation studies in bioreactors – Batch and Continuous modes

3.4.1 Elicitation studies in 2.5-L STR – Batch Mode

The effect of MO (300 mg L^{-1}), on the biomass and pH profiles, as well as bacitracin A production in *B. licheniformis* cultures was investigated. L-glutamic acid consumption, carbohydrate content, and activity of β-mannanase in the fermenters were also determined. Fermentation with no oligosaccharide supplementation was used as a control. Fermentations were carried out in parallel using identical fermenters, the same batch of inoculum and medium. The presence of MO throughout the fermentations was also investigated by TLC.

3.4.1.1 Cell growth, pH profile and L-glutamic acid consumption

No significant difference ($p \geq 0.05$) was observed in the cell growth and pH profiles between the control (Fermenter 1) and elicited cultures (Fermenter 2) (Figure 3.22).

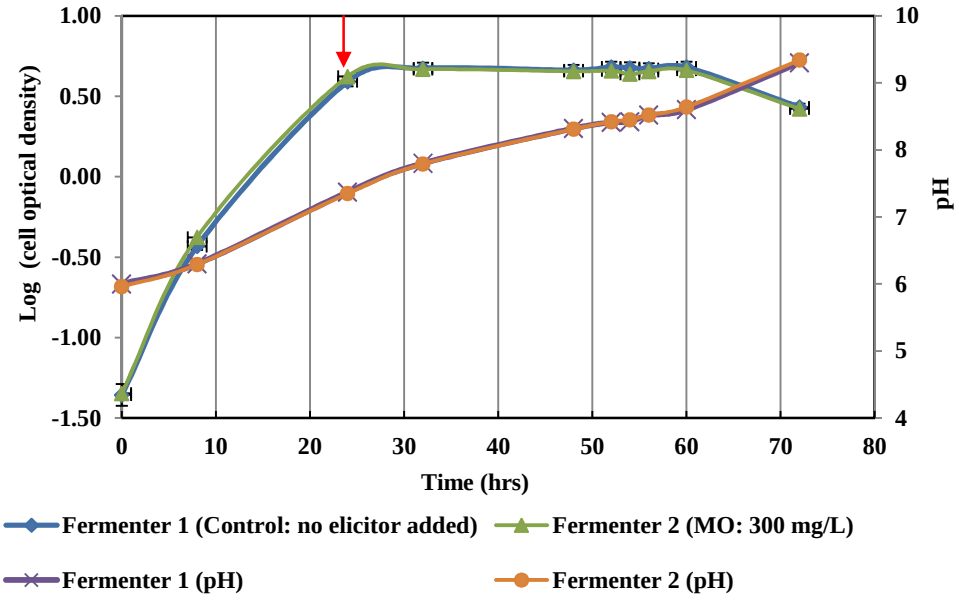


Figure 3.22 Cell growth and pH profile of 2.5-L batch fermentation of *B. licheniformis*. Fermenter 1: Control (no elicitor added), Fermenter 2: (MO: 300 mg L^{-1}) added at 24 hrs (red arrow).

The L-glutamic acid consumption rate in both control and the test fermenters was similar without any significant differences (Table 3.1). There was a decrease in the L-glutamic acid concentration as the fermentation proceeded. At 48 hrs after inoculation, L-glutamic acid was already depleted in both test and control cultures (0 g L^{-1}).

Table 3.1 L-Glutamic acid consumption rate (0 - 32 hrs) for the control and test cultures in *B. licheniformis* cultures.

Run	L-glutamic acid consumption rate (g^{-1} hrs^{-1})
Control Fermenter 1	0.43
Test Fermenter 2	0.46

3.4.1.2 Bacitracin A production

Maximum bacitracin A production of approximately 725 mg L^{-1} was observed after 52 hrs in the cultures supplemented with MO, as compared to 645 mg L^{-1} produced by the control culture (Figure 3.23). The maximum increase ($p \leq 0.05$) in the bacitracin A production was observed at 72 hrs (17 %) in the test MO elicited fermenter followed by 13 % at 48 hrs.

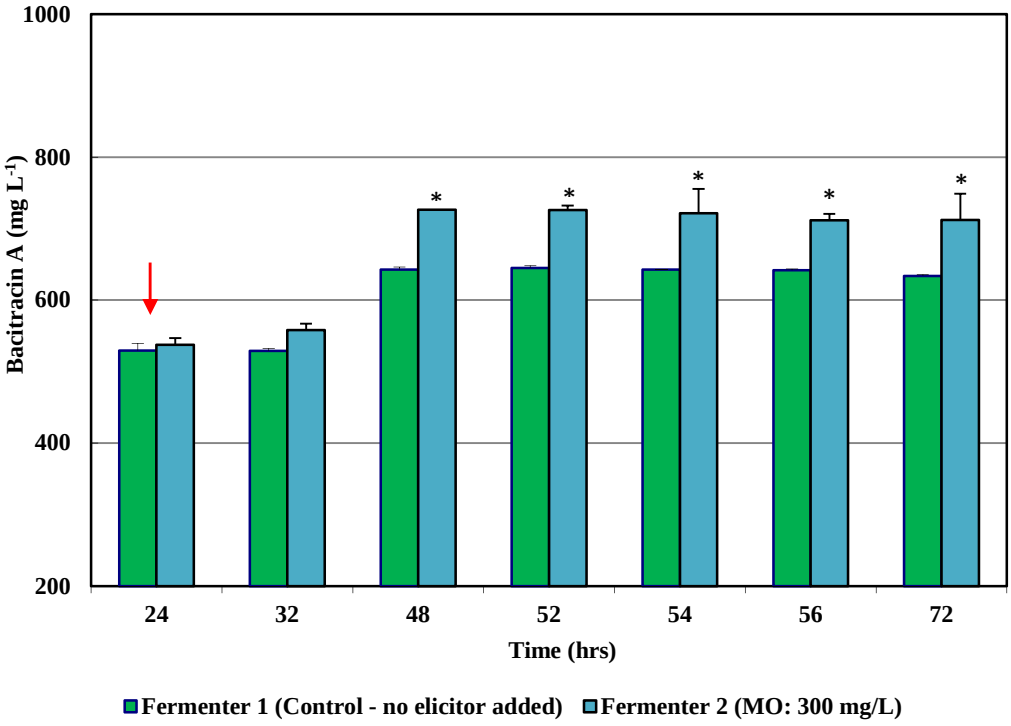


Figure 3.23 Bacitracin A production profile in cultures of *B. licheniformis* in 2.5-L STR reactor. Fermenter 1 (Control - no elicitor added), Fermenter 2: (MO: 300 mg L^{-1}) added at 24 hrs (red arrow). Error bars indicate standard deviation between triplicate samples.

3.4.1.3 β-mannanase activity

Figure 3.24 shows β-mannanase activity in batch cultures of *B. licheniformis*. The enzyme was induced in cultures as soon as MO was added to the fermenter at 24 hrs. The activity of the enzyme gradually increased during the growth. Maximum activity of approximately 19.20 U mL^{-1} was observed in cultures supplemented with MO. ß-mannanase was also produced by control culture after 48 hrs, reaching a maximum activity of 12.78 mL^{-1}. The activity of the test cultures was still significantly higher ($p \leq 0.05$) than the control culture. The highest increase of 3.5 fold was reached at 56 hrs.

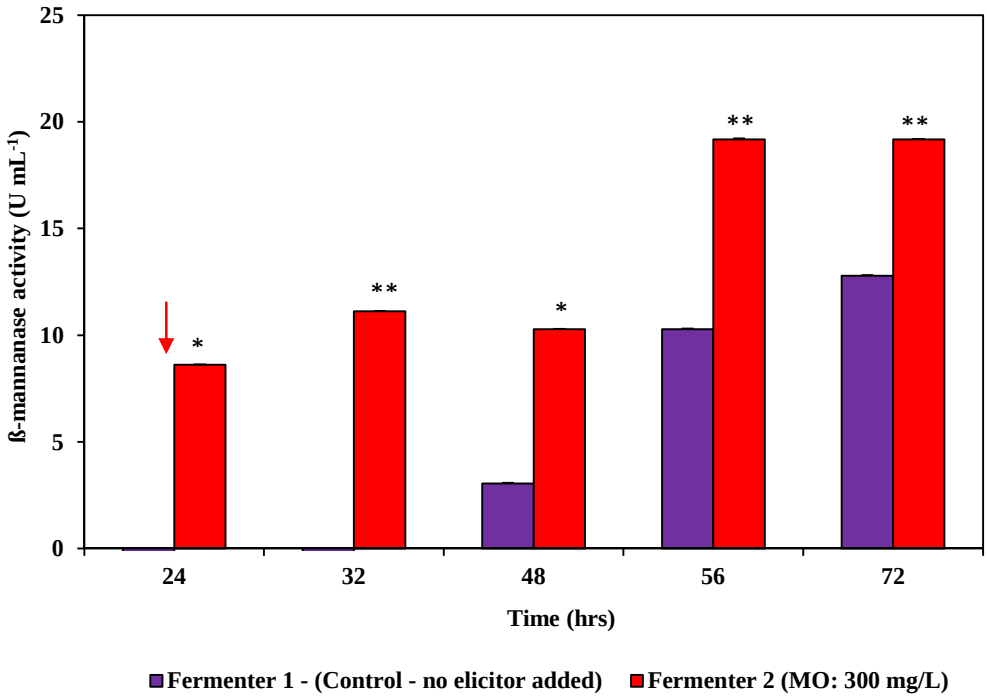


Figure 3.24 β-mannanase activity during the course of fermentation of *B. licheniformis*. Fermenter 1 (control - no elicitor added) and Fermenter 2 (MO: 300 mg L^{-1}) added at 24 hrs (red arrow). Error bars indicate standard deviation between triplicate samples.

3.4.1.4 Relationship between β-mannanase activity and total carbohydrate concentration.

Figure 3.25 shows the relationship between, β-mannanase activity and total carbohydrate concentration. β-mannanase was induced as soon as MO was added into the culture and its activity was steadily increased during the fermentation. Production of carbohydrate was observed as early as 24 hrs after inoculation, in both control and test culture. In the control culture (no elicitors added) β-mannanase activity was just observed after 48 hrs (late stationary phase), suggesting that the carbohydrate likely to induce β-mannanase is not produced till late stage of the bacterium growth. Similar results were observed in shaken flask experiments (Section 3.2.4).

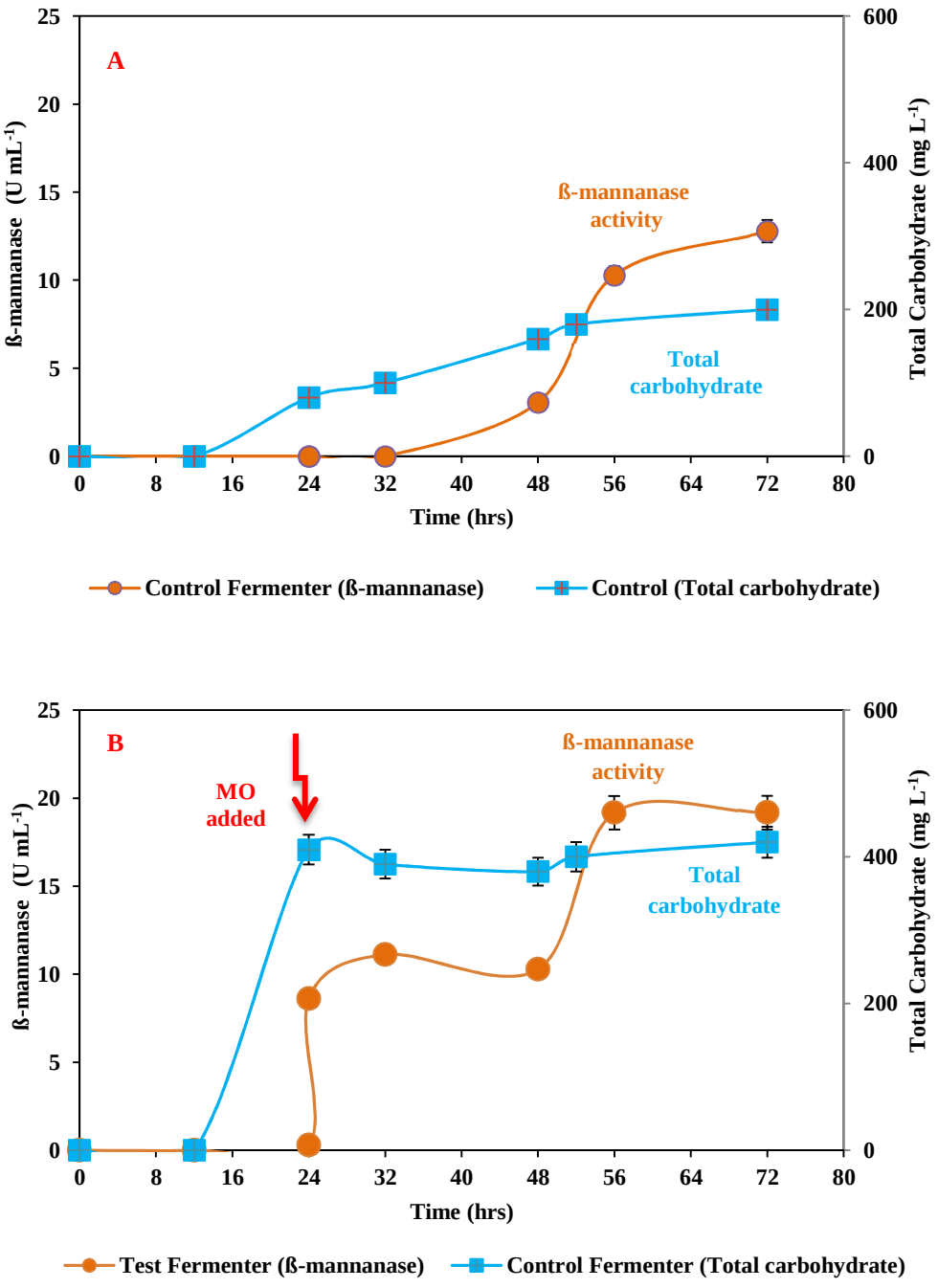


Figure 3.25 β-mannanase activity and total carbohydrate production during the course of fermentation of *B. licheniformis*. (A) Fermenter 1 (Control: no elicitor added), and (B) Fermenter 2 (MO: 300 mg L^{-1} at 24 hrs (red arrow). Error bars indicate standard deviation between triplicate samples.

3.4.1.5 Detection of MO during the Batch fermentation process

Samples of *B. licheniformis* culture were taken through the course of the fermentation. The samples were centrifuged; the supernatant was freeze dried and re-dissolved into 100 µl of distilled water. The extracts were tested for presence of MO using TLC method.

Figure 3.26 shows the presence of MO in the supernatant of the samples taken throughout the *B. licheniformis* growth. TLC results revealed that only some constituents of MO could be spotted after its addition to the culture at 24 hrs (Lanes 3). These constituents then disappeared with time (Lanes: 5 and 7). No spots were observed in the control cultures (Lanes: 2, 4, and 6).

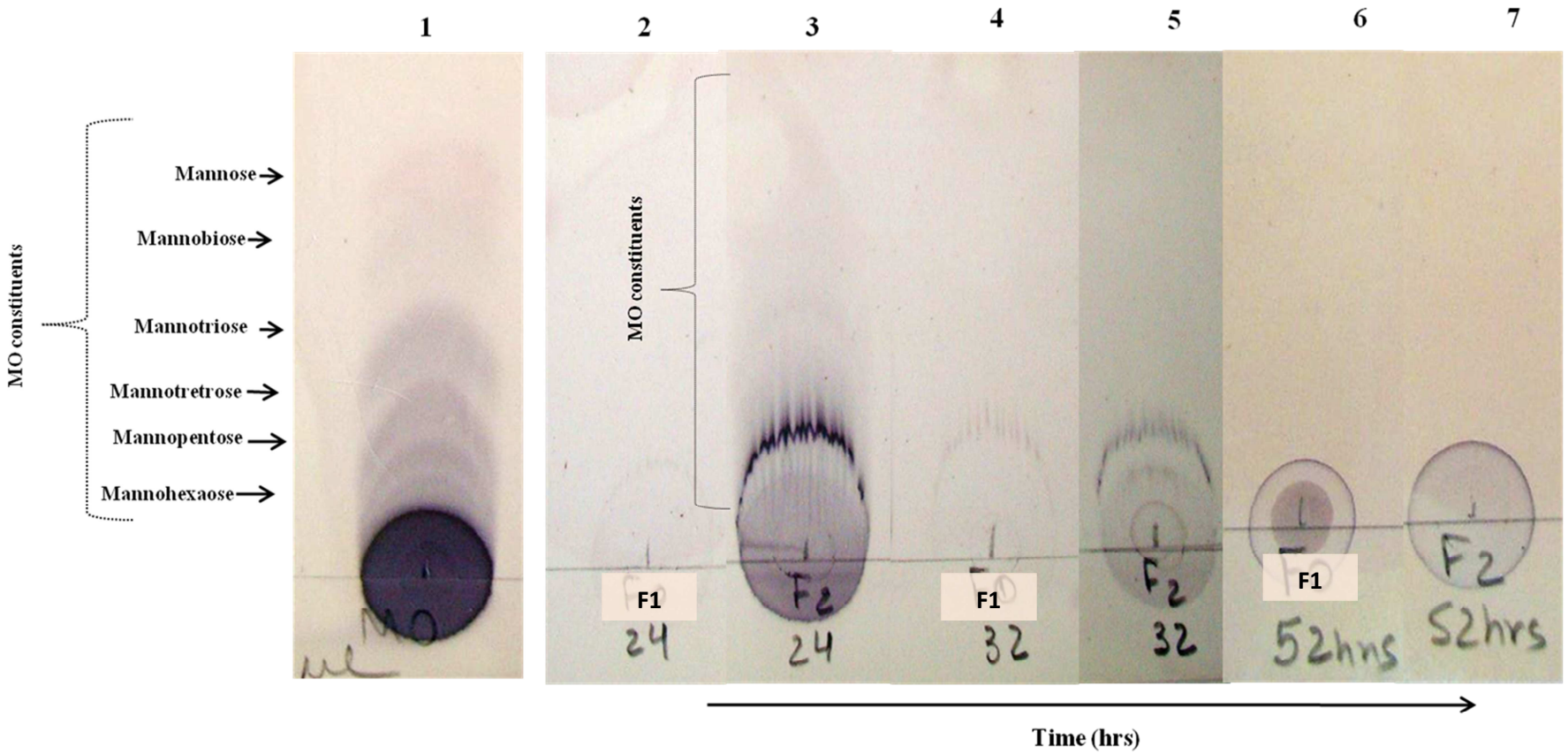


Figure 3.26 TLC profile of MO during the course of the fermentation of *B. licheniformis*. Lanes:1. Stda MO; 2. Fermenter 1: Control 24 hrs; 3. Fermenter 2: MO 24 hrs (MO: 300 mg L^{-1}); 4. Fermenter 1: Control 32 hrs (no elicitor added); 5. Fermenter 2: MO 32 hrs (MO: 300 mg L^{-1}); 6. Fermenter 1: Control 52 hrs (no elicitor added); 7. Fermenter 2: MO 48 hrs (MO: 300 mg L^{-1}). Experiments were carried out in triplicate.

3.4.2 Elicitation studies in 0.6-L STR – Continuous Mode

In order to assess if continuous MO stimulation of the culture at steady state would affect bacitracin A levels, and to examine if the effects caused by the MO elicitor observed in cultures of *B. licheniformis* are dependent on the physiological state of the cells, a continuous culture was set up at late exponential phase of growth. Biomass and pH profiles were monitored throughout the experiment as well as bacitracin A production and β-mannanase activity. MO pattern throughout the fermentation was also investigated by TLC.

3.4.2.1 Cell growth and pH profile

B. licheniformis cells were grown under batch mode up to late exponential phase (OD ~ 2.5). At this point fresh M20 medium was continuously pumped into the fermenter (F = 0.40 mL min^{-1}). Three volume changes were considered in order to obtain a steady state growth. Thereafter, a MO formulation was added to the F2 reservoir (containing M20 medium) to achieve a final concentration of 300 mg L^{-1} in the fermenter. The continuous culture was performed for 155 hrs. No significant difference ($p \geq 0.05$) was observed in the cell growth and pH profiles between the control (Fermenter 1) and the elicited cultures (Fermenter 2) before or after the MO addition (Figure 3.27).

The specific growth rate (μ = 0.23 hrs^{-1}) and doubling time (T_d = 2.97 hrs) of *B. licheniformis* cultures in 0.6-L STR fermentations were not significantly ($p \geq 0.05$) different between the control and the test cultures. After 155 hrs, the continuous mode was stopped and a batch mode was re-established.

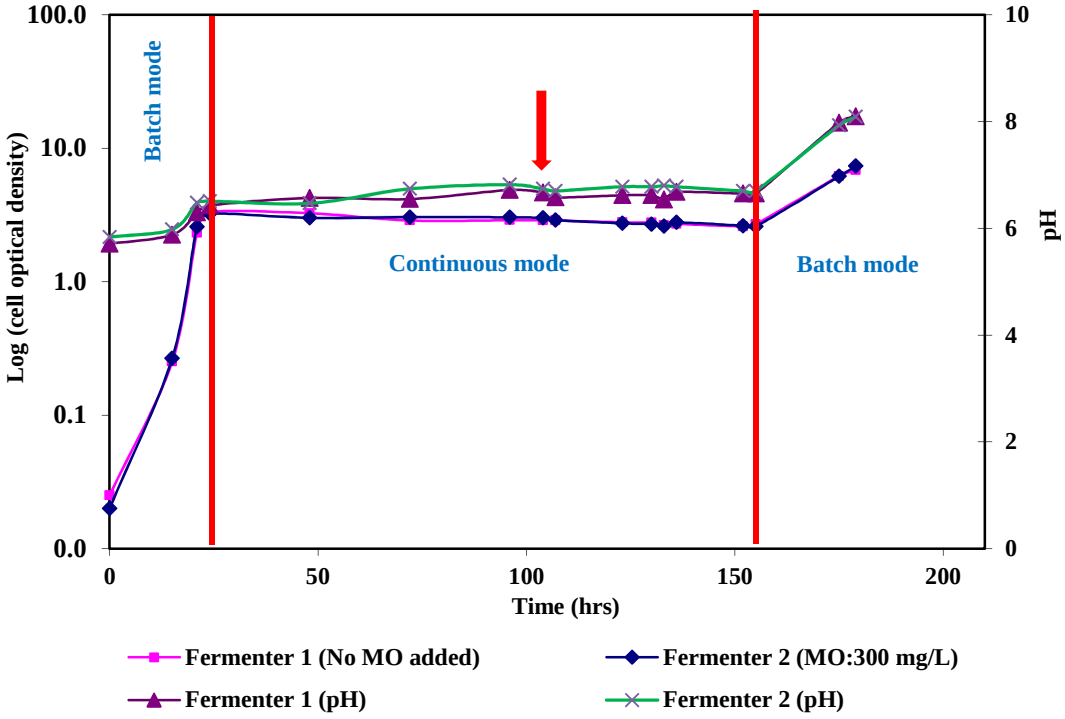


Figure 3.27 Cell growth and pH profile of 0.6-L STR continuous mode fermentation (till 155 hrs) of *B. licheniformis*. Fermenter 1: Control (no elicitor added), Fermenter 2: (MO: 300 mg L^{-1}) added at 104 hrs (red arrow). Error bars indicate standard deviation between triplicate samples.

3.4.2.2 Bacitracin A production

No significant difference ($p \geq 0.05$) was observed in bacitracin A production between the control and the treated fermenter during the continuous process (Figure 3.28). A maximum bacitracin A production of 566.64 mg L^{-1} was obtained during this time.

Figure 3.26 also shows a significant increase ($p \geq 0.05$) on bacitracin A production in the fermenter supplemented with MO, after a batch mode was re-established (175 – 179 hrs). Maximum antibiotic production of approximately 837 mg L^{-1} was observed at 179 hrs in the test bioreactor as compared to 753 mg L^{-1} in the control culture at the same time. The maximum increase in the bacitracin A production was observed at 175 hrs (17 %).

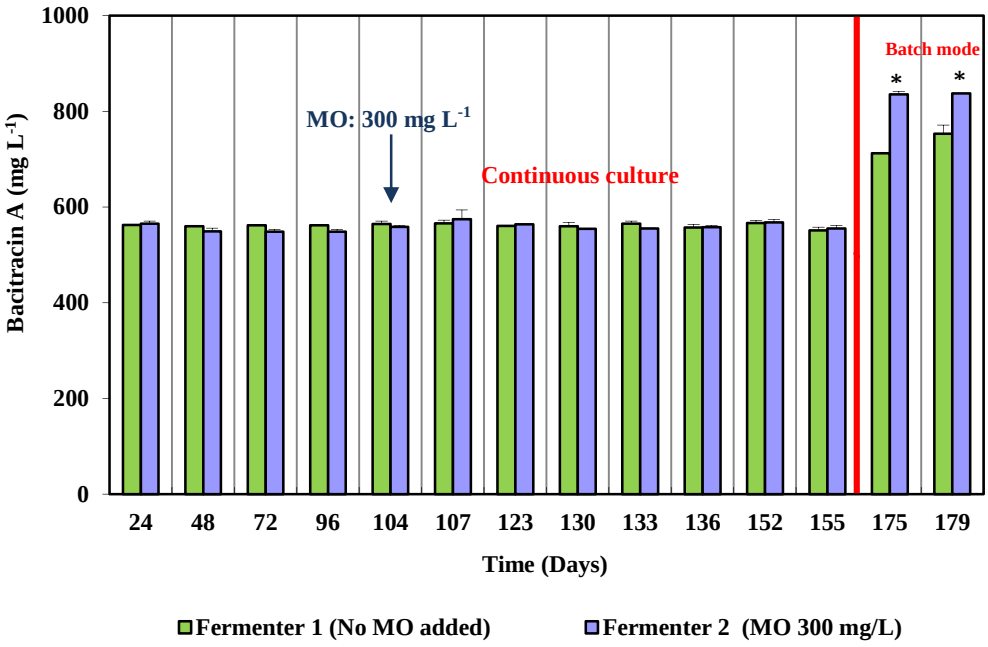


Figure 3.28 Bacitracin A production profile in cultures of *B. licheniformis*. Fermenter 1 (no elicitor added) and Fermenter 2 (MO: 300 mg L^{-1}). M20 medium was continuously pumped into the reactor till 155 hrs. Batch mode was re-established between 175 to 179 hrs. Assays were carried out in triplicates. Error bars indicate standard deviation between triplicate samples.

3.4.2.3 β-mannanase activity

Figure 3.29 shows β-mannanase activity profile in continuous cultures of *B. licheniformis*. β-mannanase activity of 2.5 U mL^{-1} was observed after M20 medium supplemented with MO started to be continuously pumped into the test fermenter (107 – 155 hrs). There was no β-mannanase activity in the control culture during the continuous mode fermentation. However, a significant increase ($p \leq 0.05$) after the change into the batch mode was observed. Highest activity of 114 U mL^{-1} was perceived in cultures supplemented with MO at 175 hrs as compared to 35.30 U mL^{-1} in control culture. An increase of 4 fold was reached at 179 hrs.

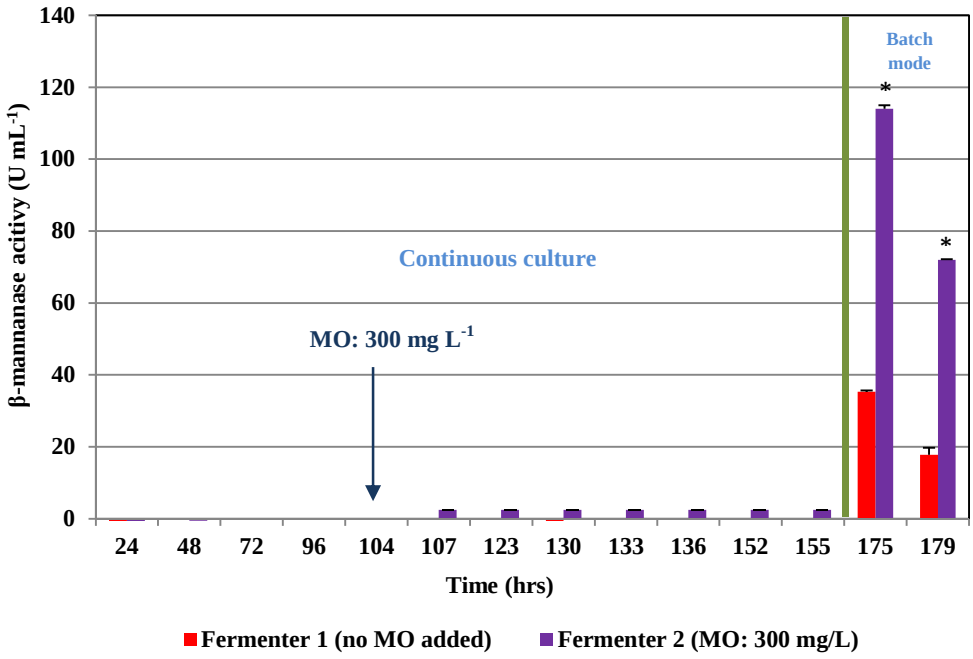


Figure 3.29 β-mannanase activity during the course of fermentation of *B. licheniformis*. Fermenter 1 (no elicitor added) and Fermenter 2 (MO: 300 mg L^{-1}) added at 104 hrs. Error bars indicate standard deviation between triplicate samples.

3.4.2.4 L-glutamic acid consumption

Figure 3.30 shows the profile of L-glutamic acid consumption. The L-glutamic acid consumption in both control and the test fermenters was similar without any significant differences. L-glutamic acid concentration kept constant throughout the continuous fermentation. However, a sharp decrease (reaching 0 g L^{-1}) was observed when batch mode was re-established at 175 hrs.

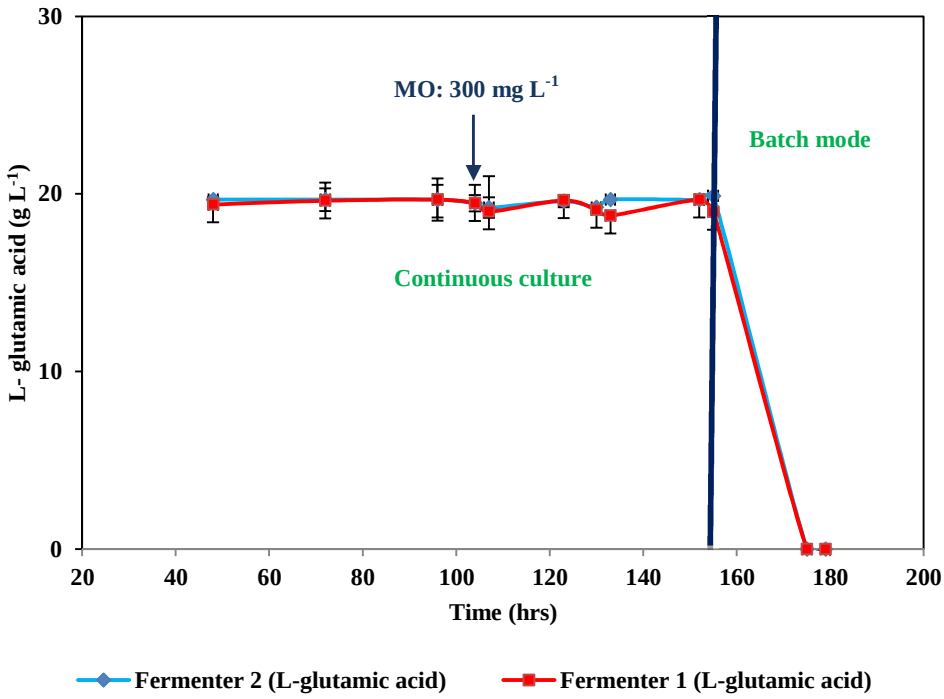


Figure 3.30 L-glutamic acid consumption during the course of fermentation of *B. licheniformis*. Fermenter 1 (Control: no elicitor added), Fermenter 2 (MO: 300 mg L^{-1} at 104 hrs). Error bars indicate standard deviation between triplicate samples.

3.4.2.5 Detection of MO during the Continuous fermentation process

Figure 3.31 shows the presence of MO detected by TLC as the continuous culture proceeded. MO presence in the medium could be observed after M20 medium supplemented with MO started to be continuously pumped into the fermenter at 104 hrs, (lane: 5) and its intensity increased as the medium supplemented with MO replaces the medium in absence of the elicitor, lanes: 5, 7 and 9, at 104, 107 and 123 hrs, respectively. However, MO disappeared after the continuous culture was stopped and a batch mode was re-established: lanes 11 and 13 at 175 and 179 hrs. No spots were observed in the control cultures (lanes: 2, 4, 6, 8, 10 and 12).

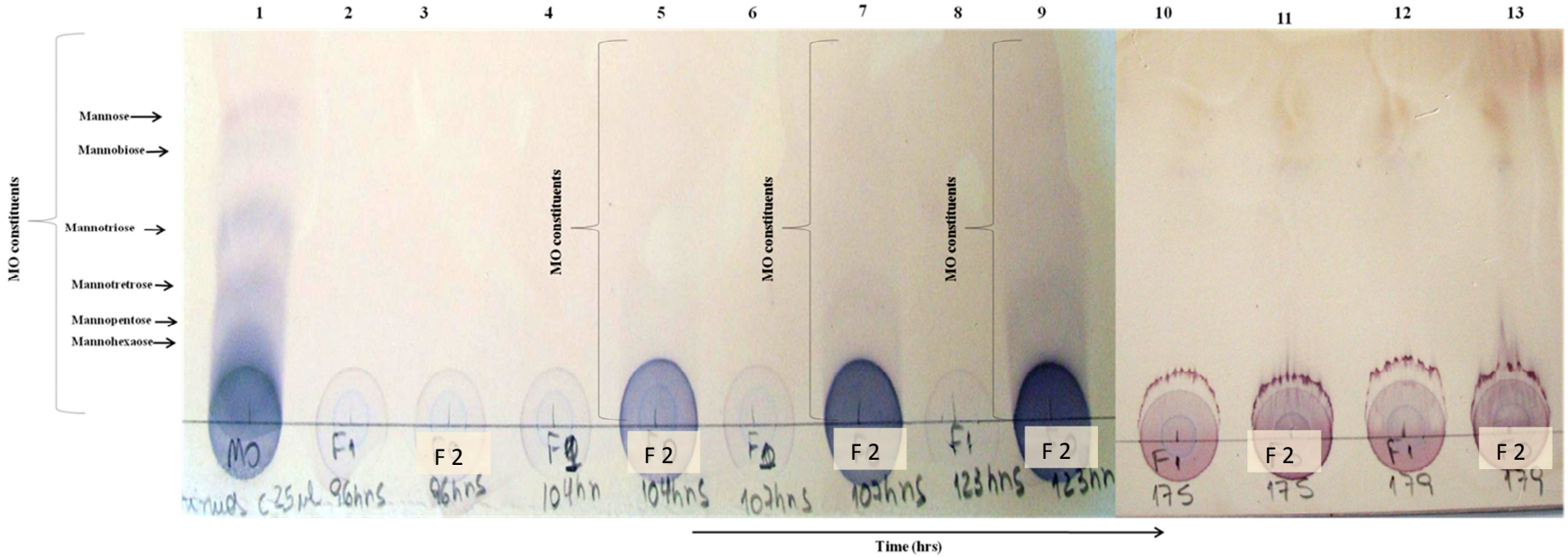


Figure 3.31 TLC profile of MO during the course of the fermentation of *B. licheniformis* during continuous culture. Lanes: 1.Standard MO; 2. Fermenter 1: at 96 hrs; 3. Fermenter 2: at 96 hrs; 4. Fermenter 1: Control 104 hrs; 5. Fermenter 2: MO 104 hrs; 6. Fermenter 0: Control 107 hrs; 7. Fermenter 2: MO 107 hrs; 8. Fermenter 1: Control 123 hrs. 9. Fermenter 2: MO 123 hrs; 10. Fermenter 1: MO 175 hrs, 11. Fermenter 2: Control 175 hrs, 12. Fermenter 1: Control 179 hrs, Fermenter 2: MO 179 hrs . Experiments were carried out in triplicate.

3.5 Measurement of intracellular Ca^{+2} levels

The effect of the oligosaccharide elicitors on the intracellular Ca^{+2} levels in *B. licheniformis* cultures was investigated as monitored by the fluorescent Ca^{+2} indicators Fluo-4. Ionomycin (ionophore) was used as a positive control. The choice of the elicitors (MO, OG and OM) and time of additions (0 and/or 24 hrs) were based on the optimisation work carried out previously (Murphy *et al*., 2007a).

Figure 3.30 shows the intracellular Ca^{+2} levels in cultures of *B. licheniformis* in response to elicitors. As soon as ionomycin, MO; OG; OM and OG/MO were added to the cultures, a significant increase ($p \leq 0.001$) in the Fluo-4 emission was detected, indicating an increase in the Ca^{+2} ion flux across the cell membrane (Figure 3.32).

Table 3.2 shows the changes observed in the intracellular Ca^{+2} levels in the supplemented cultures as compared to the control condition. An increase on Ca^{+2} influx of 9.2 % was observed in cultures treated with OG/MO followed by 9.0 % in cultures supplemented with just MO. Subsequent to the stimulation, the ionomycin treated cultures re-established their resting levels while in all elicited cultures, a significant ($p \leq 0.001$) decrease in Ca^{+2} influx across the cell membrane was observed in comparison to the control baseline.

Table 3.2 Effect of elicitors: MO, OM and OG on the intracellular Ca^{+2} levels in *B. licheniformis* cultures. Changes in Ca^{+2} influx (compared to control baseline) are shown as % difference.

	MO	**OG**	**OM**	**OG/MO**
Percentage increase	9	5	5	9.2
Percentage decrease	3	6	5.7	5

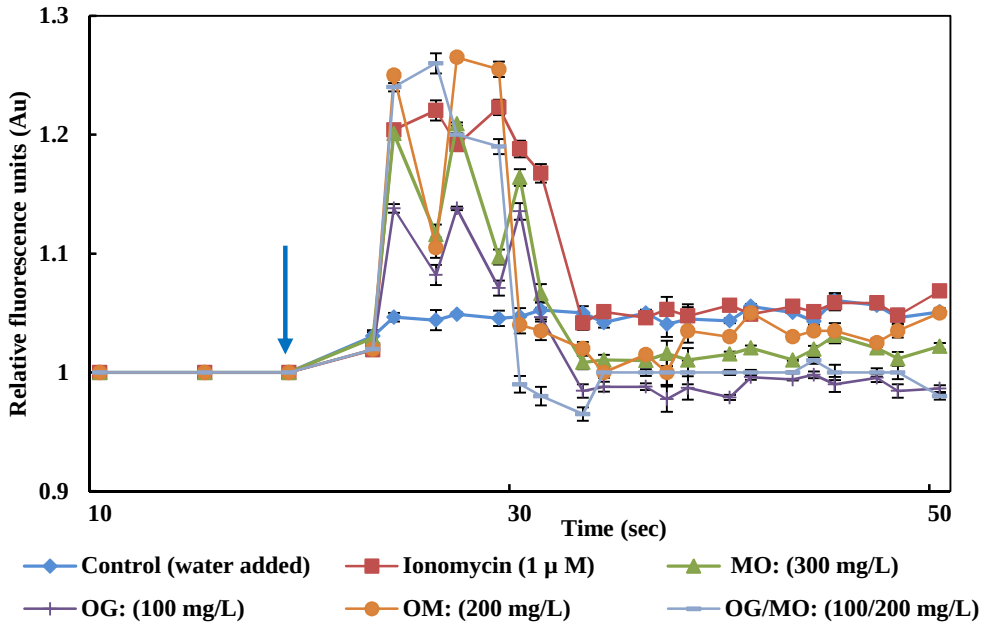


Figure 3.32 Effect of elicitor addition on the (Ca^{+2}) level in 24 hrs old cultures of *B. licheniformis*. Fluo-4 loaded cells were plated in a 12 well plate for analysis of Ca^{+2} using the FLUOstar optima system. Changes in fluorescence emission were measured. Control (water added); Ionomycin: (1 µM); MO: (300 mg L^{-1}); OG: (100 mg L^{-1}); OM: (200 mg L^{-1}) and OG: (100 mg L^{-1} at 0 hrs and MO: 200 mg L^{-1} at 24 hrs). Experiments were performed with extracellular 1 mM $CaCl_2$. Arrow represents the elicitor's addition.

To investigate whether intracellular calcium could be involved in regulating bacitracin A production, as a positive control, ionomycin was added into the culture of *B. licheniformis* at 24 hrs after inoculation and bacitracin A levels were determined (Figure 3.33).

The addition of ionomycin to the *B. licheniformis* culture increased bacitracin A production by 15 % after 32 hrs of inoculation. However, the ionomycin positive effect on bacitracin A concentration was transitory as it gradually disappeared with time (Figure 3.33). Highest bacitracin A production of 853 mg L^{-1} was detected after 52 hrs in cultures supplemented with combined OG and MO, followed by 796 mg L^{-1} in cultures treated with ionomycin and 728 mg L^{-1} by control cultures.

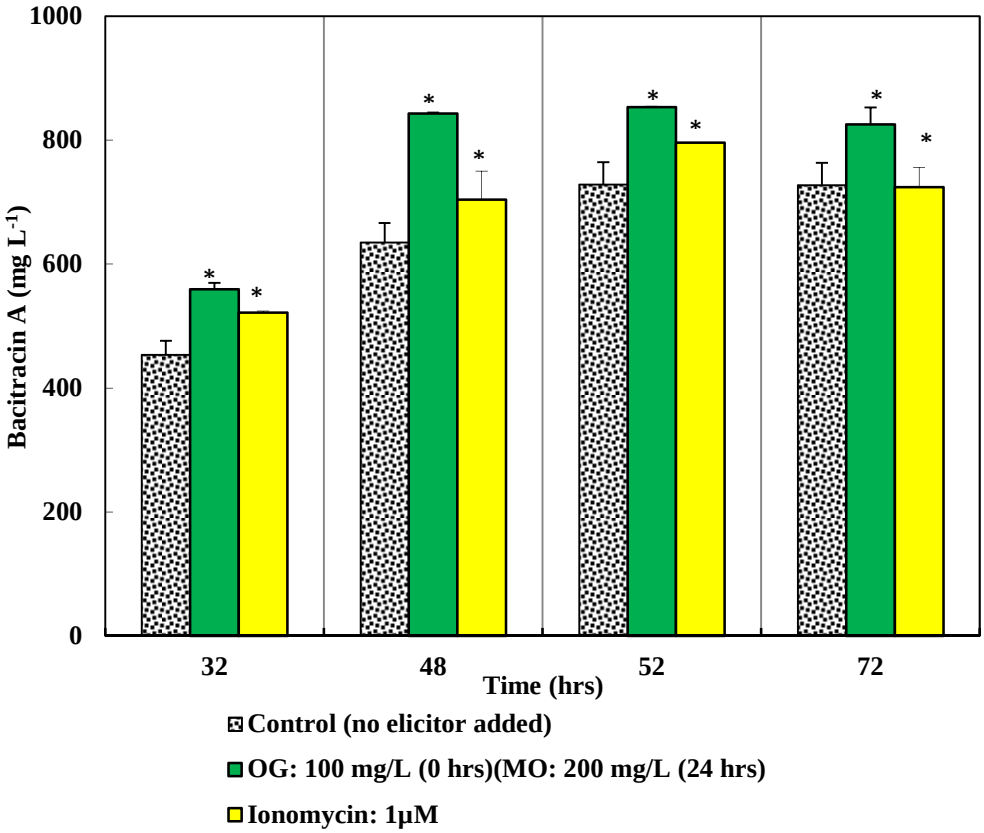


Figure 3.33 Effect of Ionomycin (1 µM) and Multiple (OG 100 mg L^{-1} and MO 200 mg L^{-1}) on bacitracin A production of *B. licheniformis*. Experiments were carried out in triplicates. Error bars indicate standard deviation between triplicate samples.

3.5.1 Effect of Ca^{2+} blocker on the biotic-elicited production of bacitracin A

It has already been established (Murphy *et al.*, 2007a) and further confirmed in this work that productivity of bacitracin A is enhanced in the presence of the selected biotic elicitors. Furthermore, this work has shown a relationship between Ca^{+2} influx and bacitracin A production (Figure 3.33). Here, we tested the effect of the Ca^{+2} channel blocker, Verapamil, on the elicitated production of bacitracin A.

3.5.1.1 Cell growth and pH

Figures 3.34 and 3.35 show the cell growth, pH and bacitracin A concentration, respectively in *B. licheniformis* cultures treated with Verapamil (calcium channel blocker) with or without the presence of the elicitors. The presence of Verapamil in cultures of *B. licheniformis* caused no significant difference ($p \geq 0.05$) in the cell growth and pH profiles between the control and the tests cultures (Figure 3.34).

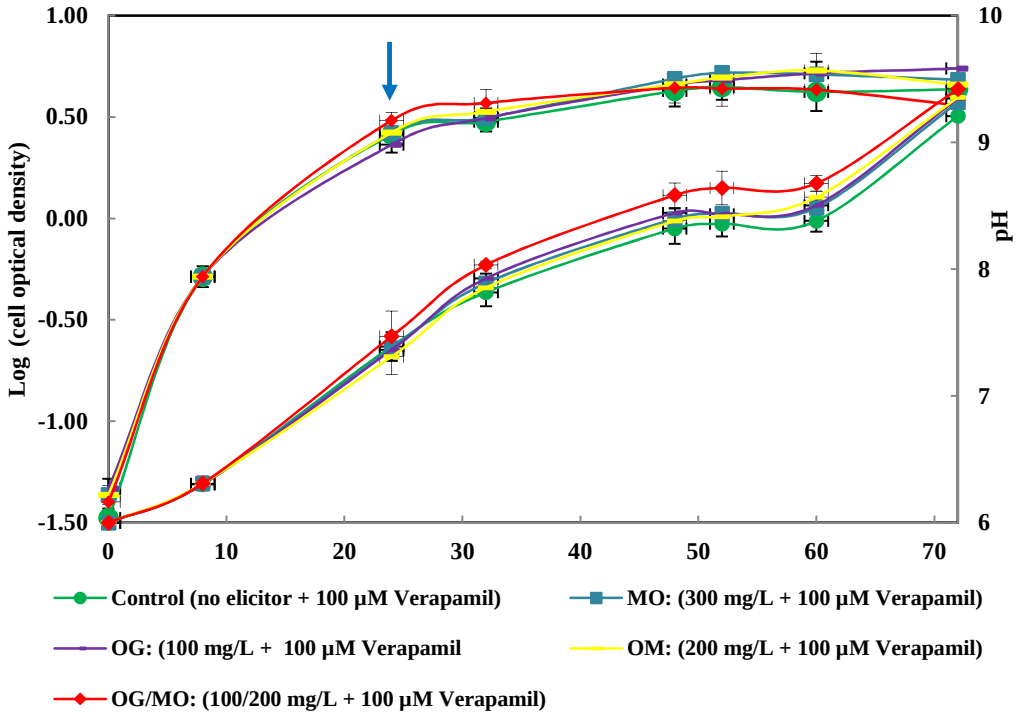


Figure 3.34 Effect of Verapamil (100 µM) in the cell growth and pH profile of control (no elicitor added) and the treated MO: (300 mg L^{-1}); OG: (100 mg L^{-1}); OM: (200 mg L^{-1}) and OG/MO (100/200 mg L^{-1}) cultures of *B. licheniformis*. Experiments were carried out in triplicate. The arrow represents the time of addition of the elicitor to the culture. Error bars indicate standard deviation between triplicate samples.

As a consequence of verapamil addition, a significant decrease ($p \leq 0.05$) in bacitracin A concentration was detected in all the cultures, including the control. When Verapamil was present in the culture broth, the highest bacitracin A concentration was approximately 243 mg L^{-1} (OG/MO added) as compared to

853 mg L^{-1} in the absence of the calcium channel blocker. Highest bacitracin A levels in the control culture was about 284 mg L^{-1} as compared to 728 mg L^{-1} under normal conditions (Figure 3.35).

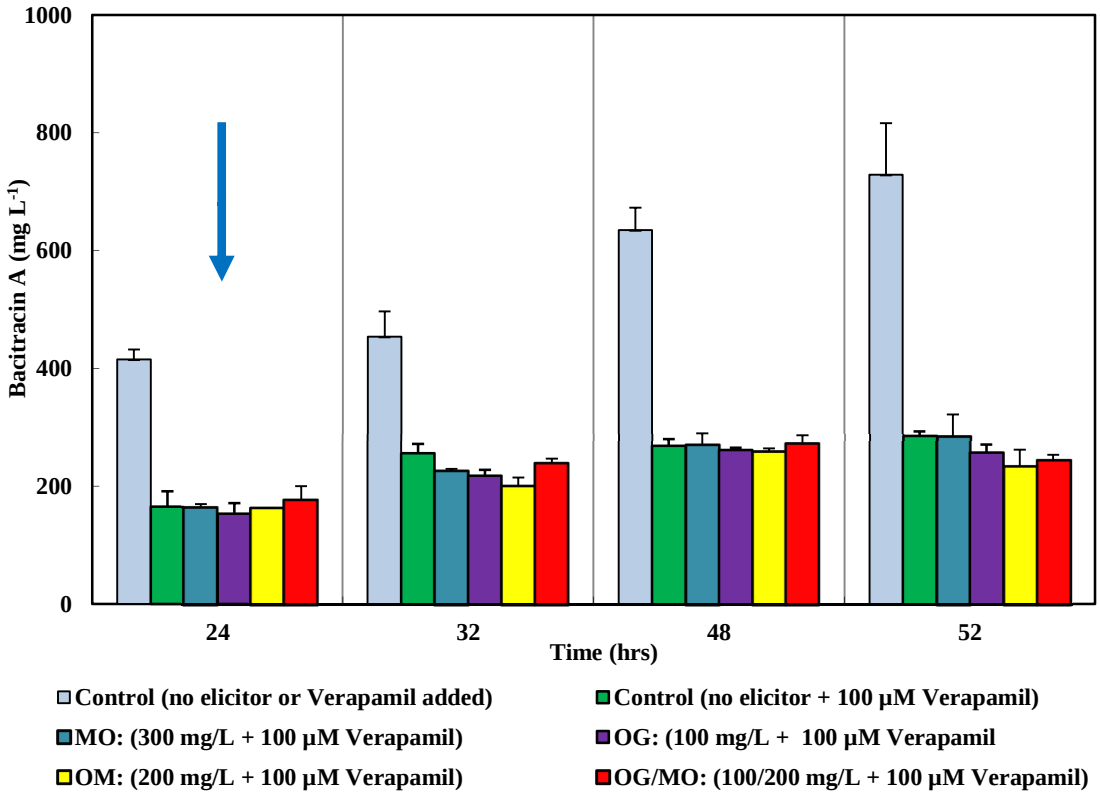


Figure 3.35 Effect of oligosaccharide elicitors on bacitracin A production in cultures of *B. licheniformis* in the presence or absence of Verapamil: Control (no elicitor or Verapamil added); Control (no elicitor + 100 µM Verapamil); MO: (300 mg L^{-1} + 100 µM Verapamil); OG: (100 mg L^{-1} + 100 µM Verapamil); OM: (200 mg L^{-1} + 100 µM Verapamil); OG: (100 mg L^{-1} and MO: 200 mg L^{-1} + 100 µM Verapamil). Experiments were carried out in triplicate. The arrow represents the time of addition of the elicitor to the culture. Error bars indicate standard deviation between triplicate samples.

Table 3.3 compares the bacitracin A concentration in the presence and absence of Verapamil in the culture broth at 48 hrs. The presence of Verapamil caused a maximum inhibition of around 74 % in the cultures treated with OM, followed by OG/MO (72 %). There was a significant decline in bacitracin A concentration of approximately (65 %) in the control cultures too.

Table 3.3 Comparison between bacitracin A production at 48 hrs, under normal elicitation conditions and under elicitor/verapamil treatment in *B. licheniformis* culture.

Conditions	Bacitracin A (mg L^{-1}) at 48 hrs of growth	
	Absence of Verapamil	Presence of Verapamil
Control (no elicitor)	634	268
MO: 300 mg L^{-1}	825	270
OG: 100 mg L^{-1}	866	261
OM: 200 mg L^{-1}	769	258
OG/MO: 100/200 mg L^{-1}	842	272

3.6 Metabolic response of *B. licheniformis* to oligosaccharide elicitors

The identification of proteins that are differentially expressed during elicitation is an important step in the understanding of the mechanism of elicitation. In this study based on previous work (Murphy, 2008), OG was added at 0 hrs and MO at 24 hrs after *B. licheniformis* inoculation and samples were taken from control and test cultures after 10 min and 24 hrs after the addition of MO (named as short-term and long-term elicitation, respectively).

A comparative proteomic analysis (2-D electrophoresis) of the intracellular *B. licheniformis* proteins in the presence and absence of elicitors uncovered differences in expression of proteins induced during short-term (Section 3.6.1), and long-term elicitation (Section 3.6.2). A brief overview of their identity is given in the following sections. The role and the significance of these proteins on the metabolic adaptation of *B. licheniformis* during elicitation will be examined in depth during the discussion section (Section 4.6.2). In total, 43 differentially expressed proteins were affected by both elicitations. The expression data for up and down-regulated proteins are summarised in Table 3.4 and 3.9, respectively. As protein phosphorylation regulates a cascade of reactions that can alter bacterial metabolism, this study also investigated the changes in the phosphoproteome pattern upon short-term elicitation (Section 3.6.1.2).

3.6.1 Proteomic profile of *B. licheniformis* supplemented cultures after short-term elicitation

3.6.1.1 Comparative pattern of whole proteome of *B. licheniformis* subjected to short-term elicitation

This section reports the results of 2-D electrophoresis to further our knowledge of the metabolic changes in *B. licheniformis* in response to short-term elicitor treatment. Figure 3.36 compares the whole proteome of *B. licheniformis* in the absence and presence of (OG/MO). Approximately 166 species were resolved between pH 3-10 of which 57 proteins from the test gels were significantly different when compared to the proteins from the control gels ($p \leq 0.05$, replicates = 6). Among these, 26 proteins (11 up-regulated, 8 down-regulated and 3 (fold change = 1.0, identification required for further discussion regards the phosphoproteins) were selected for identification by LC-MS/MS. This selection was based on the expression levels (fold change ≥ 1 or ≤ 1) and the position of the protein in relation to spots; only spots that could be excised without risk of cross mixing were selected for identification.

The normalised volumes of selected proteins and their identities, obtained by LC-MS/MS are shown in Tables 3.4 and 3.5, respectively. MS/MS spectra were submitted for search on the database in the Mascot search engine. The results summarised in Table 3.4 show the identity of the 22 spots representing 25 different proteins (as some spots contained more than one protein). Furthermore, for some proteins, two or more spots differing in their pI or MW were found to be the same protein, suggesting the presence of different isoforms or multiple forms/PTM (post translational modification) variants of the same gene product. For instance, ketol-acid reductoisomerase was detected in five places (species 1, 3, 4.3, 125 and 159) and superoxide dismutase in two (species 27 and 103) as shown in Figure 3.36. Protein phosphorylation is a very common PTM process and the occurrence of this role will be further investigated in the next section (3.6.1.2). The presence of protein isoforms with different expression patterns as a response of external stimuli has been reported in *Bacillus* spp. (Hecker and Volker, 2004).

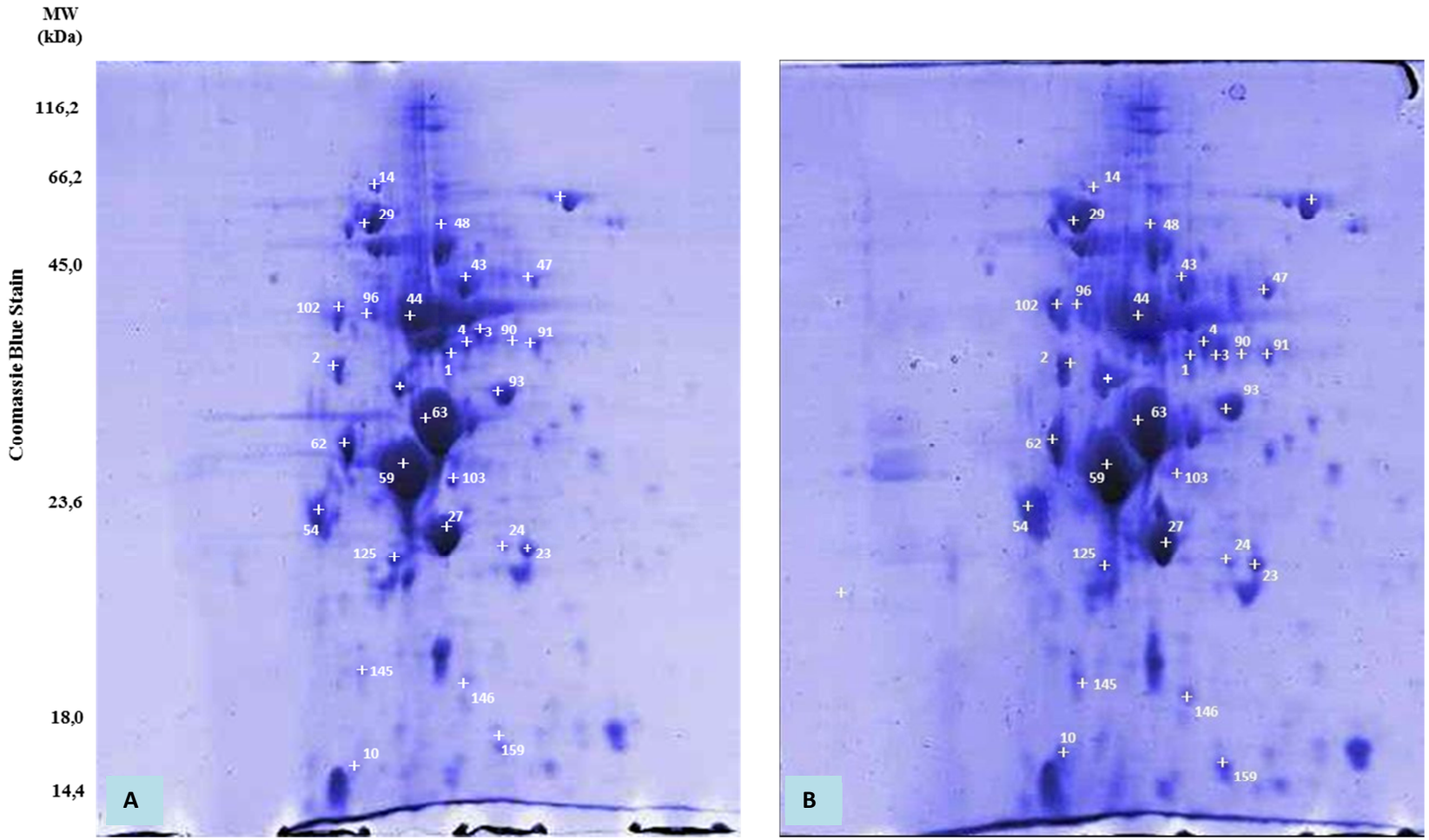


Figure 3.36 Comparative 2-DE gels showing the whole proteome of *B. licheniformis*, after short-term elicitation: (A): Control culture (no elicitor added) and (B) culture supplemented with OG at 0 hrs and MO after 24 hrs. Proteins were focussed on a pH 3-10 linear IPG strip (GE-Healthcare), separated by 2-D gels electrophoresis and stained with Coomassie Blue. Numbers represent the protein spots.

Table 3.4 Short-term OG/MO elicited proteins of *B. licheniformis*. Anova analysis generated by Progenesis Same Spots software (Non-Linear Dynamics, UK). Fold increase obtained as compared to control spots.

Spot number (refer to Figure 3.34)	Anova (*p*)	Fold change	Average Normalised Protein Volumes	
			Control	Test 10 min
Fold change = 1				
62	9.086e-005	-	4.324e+004	4.502e+004
91	3.932e-007	-	1.255e+004	1.276e+004
93	3.5.746e-004	-	2.804e+004	2.943e+004
Up-regulated - Fold change (≥ 1)				
1	1.198e-008	1.3	1.282e+004	1.663e+004
2	2.952e-013	1.9	1.748e+004	3.359e+004
3	9.780e-008	1.1	9060.787	1.015e+004
24	7.609e-007	1.3	4578.736	5835.000
44	3.373e-011	1.3	1.050e+005	1.366e+005
47	8.845e-012	1.9	8144.884	1.526e+004
54	0.0001	1.3	8987.425	1.175e+004
102	9.389e-010	1.7	2.807e+004	4.641e+004
146	1.110 e-010	1.5	4209.52	6355.0
159	0.0001	1.3	8987.425	1.175 e+004
145	2.922 e-009	1.4	5774.139	8235.00
Down-regulated – Fold change (≤ 1)				
4	1.858e-009	0.86	4230.043	3629.000
14	1.416e-008	0.65	7213.910	4697.00
27	3.495e-013	0.87	3.532e+004	3.065e+004
48	4.094e-008	0.76	6006.738	4572.0
59	1.422e-013	0.82	1.344e+005	1.105e+005
96	8.910e-012	0.81	6554.478	5313.000
103	1.488e-014	0.40	4940.612	1942.000
125	1.574e-011	0.54	2972.909	1615.000

Table 3.5 Short-term elicited proteins of *B. licheniformis*. Characteristics of the protein spots separated by two-dimensional electrophoresis, detected by Coomassie Blue and identified by LC-MS/MS.

Spots number / putative protein	Accession number[a)]	Gene	Protein function	Approx. MW (kDa)/ pI	Sequence coverage (%)	LC-MS/MS	
						Mascot Score	N° of peptides[b)]
Fold change (= 1)							
62 Hypothetical protein BL01967	52080921	*3027934*	Periplasmic transport system	34.9/5.1	18.2	373.1	5
91 Phosphoserine aminotransferase (PSAT)	52079489	*Serc*	L-serine biosynthesis	39.6/5.9	17.6	206.9	4
93 Hypothetical protein BL0047493	52081194	*BL00474*	Tyrosine metabolism	34.7/5.6	7.3	154.9	2
Up-regulated - Fold change (≥ 1)							
1 Ketol-acid reductoisomerase	52081312	*ilvC*	Valine and leucine biosynthesis	37.4/5.4	11.1	192.9	3
3 Ketol-acid reductoisomerase	52081312	*ilvC*	Valine and leucine biosynthesis	37.4/5.4	16.1	281.1	4
159 Ketol-acid reductoisomerase	52081312	*ilvC*	Valine and leucine biosynthesis	37.4/5.4	6.7	147.8	2
47.3 3-phosphoshikimate 1-carboxyvinyltransferase	52786139	*aroE*	Aromatic amino acid biosynthesis	45.5/5.9	5.1	173.3	1
24 Translaldolase	52082245	*tal*	Pentose phosphate pathway	22.8/5.8	38.9	501.7	6
102 Phosphopyruvate Hydratase	52081958	*Eno*	Glycolysis	46.6/4.5	12.8	289.8	4

145 Nucleoside diphosphate kinase	52080782	*Ndk*	Cellular metabolism	16.7/5.0	41.9	418.3	6
2.1 NAD synthetase	52078821	*nadE*	General stress protein	30/4.8	14.0	211.7	3
146 Thioredoxin domain-containing protein	52080022	*ykuV*	Stress response	17.5/5.3	13.2	84.7	2
54.1 Stress response protein YceD	52078807	*yceD*	Stress Response	20.8/4.3	4.3	237.7	4
2.2 Translocation dependent antimicrobial spore component	52080998	*tasA*	Sporulation	28.8/5.3	8.7	133.8	2
44 Oxalate decarboxylase	52081879	*oxdC*	Sporulation	43.5/5.0	8.1	222.0	3
Down-regulated – Fold decrease (≤ 1)							
4.1 Bifunctional 3-deoxy-7-phosphoheptulonate synthase/chorismate mutase	52081475	*aroA*	Aromatic amino acid biosynthesis	39.7/5.4	12.3	243.6	3
125 Ketol-acid reductoisomerase	16079881	*ilvC*	Valine and leucine biosynthesis	37.4/5.4	12.9	193.3	3
4.3 Ketol-acid reductoisomerase	16079881	*ilvC*	Valine and leucine biosynthesis	37.4/5.4	6.7	93.9	2
4.2 Enoyl-CoA hydratase	152975512	*Bcer98_1732*	Fatty acid metabolism	39.1/5.2	2.6	57.0	1
14.2 Chaperone protein DnaK	580853	*dnaK*	Stress response	23.1/4.5	6.5	58.8	1
27 Superoxide dismutase	52081038	*sodA*	Oxidative stress	22.5 /5.2	33.7	541.9	4

103 Superoxide dismutase	52081038	*sodA*	Oxidative stress	22.5/5.2	17.3	231.4	3
96 Alkyl hydroperoxide reductase	52080021	*ykuU*	Oxidative stress	20.4/4.4	15	205.9	2
48.2 Oxalate decarboxylase	52081879	*oxdC*	Sporulation	43.5/5.0	5.0	90.0	2
59 Translocation dependent antimicrobial spore component	52080998	*tasA*	Sporulation	28.8/5.3	22	362.3	4

a) Accession numbering (entries in NCBInr).
b) The sequenced peptides identified by LC-MS/MS are indicated below:
Spot 1: K.ESGVDVIVGVR.K, K.AEEDGHQVFTVR.E, K.LIVDLMYEEGLAGMR.Y.
Spot 2 (1): K.GFVLGISGGQDSTLAGR.L, R.LAQLAVEELR.E, R.SLLEELGAPER.L.
Spot 2(2): K.AIEPEFLHDNGK.I, K.VQLVIEFVNDK.T.
Spot 3: K.ESGVDVIVGVR.K, K.AEEDGHQVFTVR.E, K.LIVDLMYEEGLAGMR.Y, R.YSISDTAQWGDFVSGPR.V.
Spot 4 (1): M.SNTELDLLR.Q, R.TSPYDFQGLGVEGLQILK.R, K.QETHLPVFVDVTHSTGR.R.
Spot 4 (2): K.ESGVDVIVGVR.K, K.AEEDGHQVFTVR.E
Spot 4 (3): R.HDDFFEGVR.S
Spot 14 (1): K.LGEHEFNR.K, K.TREDLAVVVQQQLK.E
Spot 14 (2): K.AVITVPAYFNDAER.Q
Spot 23: K.ENISFHDR.L, K.TNVTLIFSANQALLAAR.A, R.AGATYVSPFLGR.L, R.LDDIGHNGLDLISEIK.Q, K.QIFDVHGLDTQIIAASIR.H, R.HAQHVTEAALR.G
Spot 44 (1): K.IPHNVER.D, K.FSFSDVHNR.L, K.TIASALVEVEPGGIR.E.
Spot 44 (2): R.MTVFASDGHAR.T
Spot 47: K.GLDQLAEPAELLDVGNSGTTIR.L
Spot 48 (1): K.LAALIILDGFGLR.D, R.AIQISNTFTNDDFR.D
Spot 48 (2): K.FSFSDVHNR.L, K.TIASALVEVEPGGIR.E
Spot 54 (1): K.ISFVITIHEAEAR.R, R.VINEDSNEELIR.Y, R.YDLGEDFSIETALITGELYR.H, K.FSAIGSGYQGGLAR.I
Spot 54 (2): K.NAQDGISLIQTAEGALTETHSILQR.V, R.LEHTINNLGASSENLTAAESR.I
Spot 93: K.EFGPELYETGETR.I, R.SGDTIHTAPR.A
Spot 103: K.SVEELVANLDAVPENIR.T, K.FGSFDQFK.E, K.EDFAAAAAGR.F
Spot 125: K.LIVDLMYEEGLAGMR.Y, R.YSISDTAQWGDFVSGPR.V, K.EWIVENQVNRPR.F
Spot 145: K.TFVMVKPDGVQR.Q, R.QLIGEILLR.F, K.VSEETAGEHYK.E, R.GDFGMFVGK.N, K.NIIHGSDSPESAAR.E, R.EINLFFK.E.
Spot 146: K.EAMPQVNEFR.D, K.LNVVAVHMPR.S
Spot 159: K.ESGVDVIVGVR.K, K.AEEDGHQVFTVR.E

3.6.1.1.1 Summary of short-term elicited proteins of *B. licheniformis*

The functional grouping of the identified up-regulated and down-regulated proteins affected by combined OG and MO treatment in *B. licheniformis* cultures showed a complex pattern as seen in Figure 3.37. The significance of these findings will be further discussed in Chapter 4.

After short-term elicitation, translaldolase and enolase two key regulatory enzymes of the pentose phosphate pathway and glycolysis, respectively, were up-regulated. Up-regulation of these energy generation enzymes may be as a result of an increased energy requirement to sustain the amplified metabolic activity caused by the elicitors. Furthermore, seven proteins involved in amino acid biosynthesis (4 up-regulated and 3 down-regulated) were differently expressed in *B. licheniformis,* implying that variation of novel protein biosynthesis or that by-products from these pathways, might be part of the adaptation process of the bacterium to the short-term elicitation of OG and MO. The results also suggest that OG and MO might have been acting as stress factors, since proteins involved in sporulation (3 up-regulated and 1-down regulated) and general stress response (1 up-regulated) and antioxidant enzymes reported to maintain ROS levels, superoxide dismutase (2 down-regulated), alkyl hydroperoxide (1 down-regulated) were also differentially expressed in *B. licheniformis* after short-term elicitation.

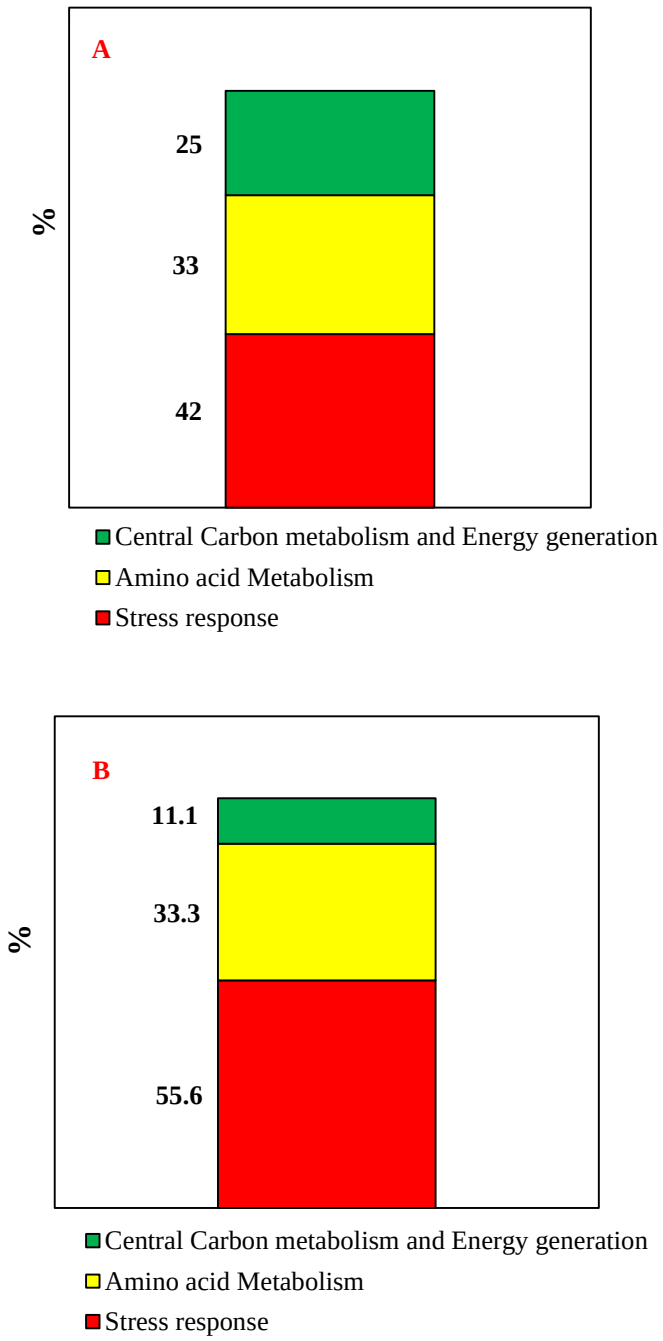


Figure 3.37 Functional grouping of the cytosolic *B. licheniformis* proteins affected by the short treatment of OG/MO. (A) up-regulated proteins and (B) down-regulated proteins.

3.6.1.2 Comparative pattern of phosphoproteome of *B. licheniformis* subjected to short-term elicitation

Protein phosphorylation of serine/threonine/tyrosine residues can regulate a cascade of reactions that can alter bacterial metabolism. Potential PTM (post translational modifications) was observed to be occurring with proteins identified (i.e. oxalate decarboxylase and ketol-acid reductoisomerase) in the previous section 3.6.1.1. We decided to investigate the changes in the phosphoproteome pattern of *B. licheniformis* upon short-term elicitation (10 min after MO addition at 24 hrs).

In order to explore the effect of the elicitors on the phosphorylation rate of the proteins, a preliminary experiment was carried out with samples taken 5, 10, 20 and 30 min after addition of MO to *B. licheniformis* cultures. Proteins were extracted, separated according to their pI and size by 2-D electrophoresis, and stained with a phosphodye (Pro-Q Diamond). The intensity of phosphoproteins detected on the test gels were higher in the 10 min test (results not shown). Therefore, our phosphoproteome analysis was carried out based on a 10 min test. Proteins extracted from the cells grown without elicitor were used as a control.

Pro-Q Diamond stain binds specifically to proteins with phosphate groups on serine, threonine, and tyrosine residues. Because of its fluorescent nature, Pro-Q Diamond can detect phosphorylated proteins present in as low as 4 ng per spot. The staining intensity correlates with the number of phosphate groups present in the respective proteins. This high sensitivity is critical in the case of phosphorylated proteins because of their very low abundance *(*Jacob and Turch, 2008). Pro-Q Diamond staining allows the comparative expression profiling of the phosphoproteome both in a quantitative and qualitative manner. Furthermore, the dye binds noncovalently to the phosphate groups, and is thus compatible with subsequent mass spectrometric analysis.

Figure 3.38 compares the phosphoproteome of *B. licheniformis* after short-term elicitation. Approximately 93 species were detected. The 12 polypeptides that showed alterations in their phosphorylation levels, as compared to the control gels, were analysed by Progenesis Same spot software and identified by LC-

MS/MS. Tables 3.6 and 3.7 show the normalised volumes of the selected proteins and their identities. MS/MS spectra were searched using the Mascot search engine.

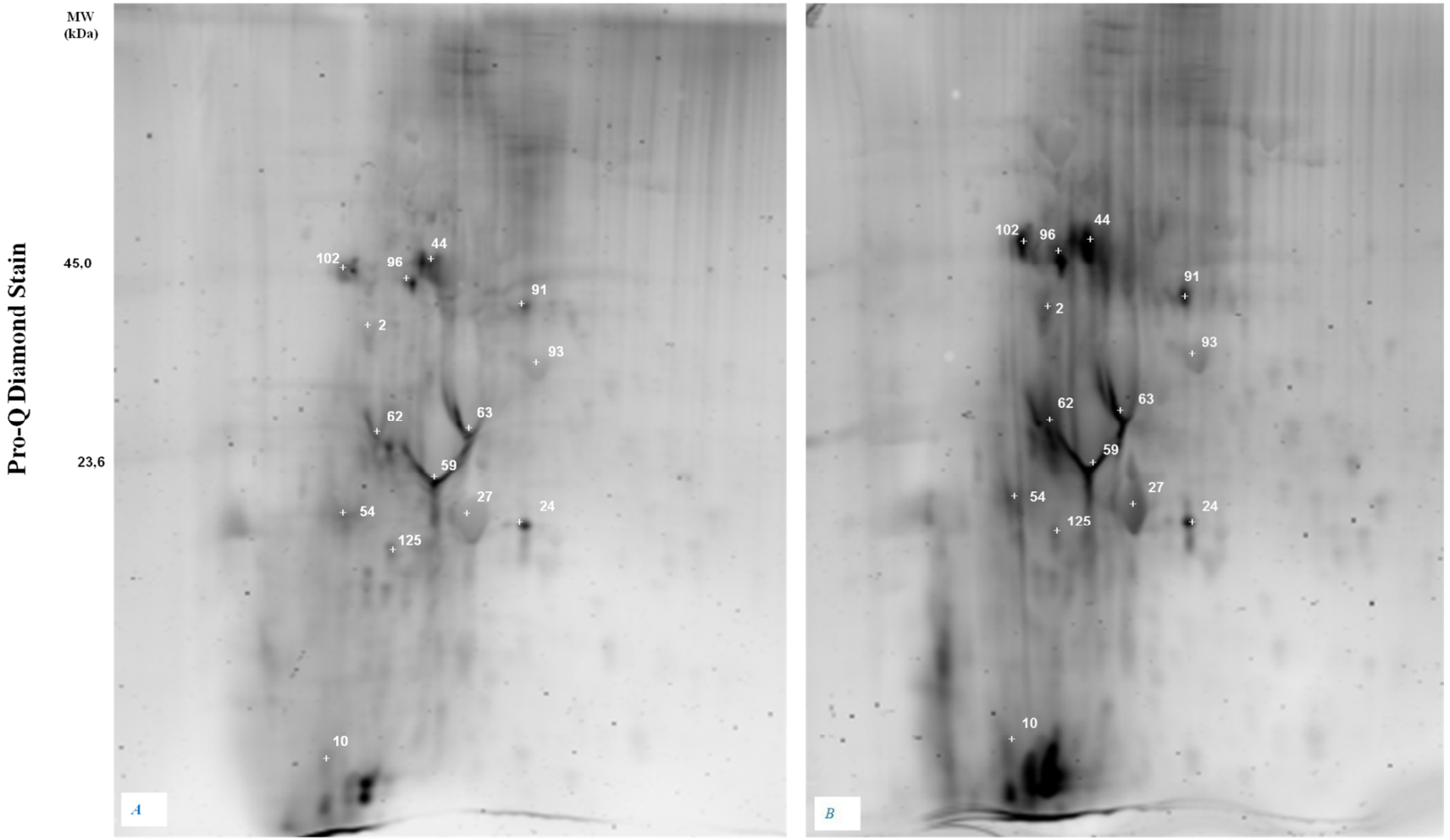


Figure 3.38 Comparative 2-DE analyses showing the phosphoproteome of *B. licheniformis* after short-term elicitation. (A) Control (no elicitor added) (B) multiple elicitors OG (0 hrs) and MO (24 hrs). Proteins were focussed on a pH 3-10 linear IPG strip (GE-Healthcare), separated by 2-D gels electrophoresis and stained with Pro-Q Diamond phosphoprotein stain (Invitrogen). Numbers represents the protein spots.

Table 3.6 Phosphorylated proteins of *B. licheniformis* after short-term elicitation with OG and MO**.** Anova analysis generated by Progenesis Same Spots software (Non-Linear Dynamics, UK). Fold increase obtained as compared to control spots.

Spot number (Refer to Figure 3.36)	Anova (*p*)	Fold change	Average Normalised Protein Volumes	
			Control	Test 10 min
2	0.021	1.9	2530.98	4926.56
24	0.043	1.5	1576.50	2408.90
27	0.015	2.1	5374.30	11157.80
44	0.006	1.8	8125.20	14613.90
54	0.042	1.7	8033.20	13584.30
59	0.005	2.4	2682.40	6534.70
62	0.042	1.7	8033.20	13584.30
91	0.011	1.9	2547.20	4862.60
96	0.013	2.1	1928.30	3996.80
93	0.043	1.3	2957.53	2249.51
102	0.018	1.9	4912.20	9436.90
125	0.016	1.8	518.88	938.444

Table 3.7 Short-term elicited phosphoproteins of *B. licheniformis*. Characteristics of the proteins separated by two-dimensional electrophoresis, detected by Pro-Q Diamond and identified by LC-MS/MS.

Spots number / putative protein	Accession number[a)]	Gene	Protein function	Approx. Mw (kDa)/ pI	Sequence coverage (%)	LC-MS/MS	
						Mascot Score	Nº of peptides[b)]
91 Phosphoserine aminotransferase (PSAT)	52079489	*Serc*	L-serine biosynthesis	39.6/5.9	17.6	206.9	4
93 Gentisate 1,2-dioxygenase	52081194	*BL00474*	Tyrosine metabolism	34.7/5.6	7.3	154.9	2
125 Ketol-acid reductoisomerase	16079881	*ilvC*	Valine and leucine biosynthesis	37.4/5.4	12.9	193.3	3
2 .1 NAD synthetase	52078821	*nadE*	NAD biosynthesis	30/4.8	14.0	211.7	3
24 Transaldolase	52082245	*talA*	Pentose phosphate pathway	22.8/5.8	30.3	331.1	5
102 Phosphopyruvate Hydratase	52081958	*Eno*	Glycolysis	46.6/4.5	12.8	289.8	4
44.1 Oxalate decarboxylase	52081879	*oxdC*	Sporulation	43.5/5.0	8.1	222.0	3
44.2 Oxalate decarboxylase	52081879	*oxdC*	Sporulation	43.5/5.1	5.2	153.4	1
59 Translocation dependent antimicrobial spore component	52080998	*tasA*	Sporulation	28.8/5.3	22	362.3	4

2.2 Translocation dependent antimicrobial spore component	52080998	*tasA*	Sporulation	28.8/5.3	8.7	133.8	2
27 Superoxide dismutase (SOD)	52081038	*sodA*	Oxidative stress	22.5 /5.2	33.7	541.9	4
96 Alkyl hydroperoxide reductase (Ahp)	52080021	*ykuU*	Oxidative stress	20.4/4.4	15	205.9	2
54.1 Stress response protein YceD	52078807	*yceD*	Stress response	20.8/4.3	4.3	237.7	4
62 Hypothetical protein BL01967	52080921	*3027934*	Hypothetical protein	34.9/5.1	18.2	373.1	5

a) Accession numbering (entries in NCBInr).
b) The sequenced peptides identified by LC-MS/MS are indicated below:
Spot 2: K.GFVLGISGGQDSTLAGR.L, R.LAQLAVEELR.E, R.SLLEELGAPER.L.
Spot 24: K.ENISFHDR.L, K.TNVTLIFSANQALLAAR.A, R.AGATYVSPFLGR.L, R.LDDIGHNGLDLISEIK.Q, R.HAQHVTEAALR.G.
Spot 27: K.SVEELVANLDAVPENIR.T, K.FGSFDQFKEDFAAAAAGR.F, K.TPILGLDVWEHAYYLNYQNR.R, K.AFWNVVNWDEVAR.L.
Spot 44 (1): K.IPHNVER.D, K.FSFSDVHNR.L, K.TIASALVEVEPGGIR.E.
Spot 44 (2): R.MTVFASDGHAR.T
Spot 54 (1): K.ISFVITIHEAEAR.R, R.VINEDSNEELIR.Y, R.YDLGEDFSIETALITGELYR.H, K.FSAIGSGYQGGLAR.I
Spot 59: K.EVLMQVGYSNFVDGNAK.N, K.NIILDEANLYDLYNMSAK.K, K.AIEPEFLHDNGK.I, K.VQLVIEFVNDK.T.
Spot 62: K.GEAEVAFSQTDIATYAIEGK.E, K.AIAALYPETVQIVTTEK.S, K.YPFYAEDTIK.K, K.YPFYAEDTIKK.G, K.ALFEADKINHAK.G.
Spot 91: K.LFGNTSIIASSK.E, K.FPDAPIPVVADMSSDILSR.K, R.KIDVSQFDIIYAGAQK.N, K.AGILYNAIDESGGFYR.G.
Spot 93: K.EFGPELYETGETR.I, R.SGDTIHTAPR.A
Spot 96: K.YPLAADTNHEVSR.E, R.EYGVLIEEEGVALR.G.
Spot 102: M.PYIVDVYAR.E, R.GNPTVEVEVYTESGAFGR.A, R.ALVPSGASTGEYEAVELR.D, R.AGYTAVISHR.S.
Spot 125: K.LIVDLMYEEGLAGMR.Y, R.YSISDTAQWGDFVSGPR.V, K.EWIVENQVNRPR.F

3.6.1.2.1 Summary of short-term elicited phosphoproteins of *B. licheniformis*

The functional grouping of the identified phosphoproteins affected by combined OG and MO treatment in *B. licheniformis* cultures are shown in Figure 3.39.

Fourteen proteins, with a significant fold increase in phosphorylation levels, were identified. Examples include enzymes involved in protection of the intracellular environment from oxygen, superoxide dismutase, SOD, (spot 27) and alkyl hydroperoxide reductase (spot 96). Additionally a translocation dependent antimicrobial spore component (spot 59) known to be stressed and phosphorylation controlled (Tam *et al*., 2006) was one of the proteins with increased phosphorylation. This implies that the activity of these stress response proteins is regulated via phosphorylation. Furthermore, the activity of two key glycolytic enzymes, phosphopyruvate hydratase (spot 102) and putative transaldolase (spot 24), and phosphoserine aminotransferase (spot 91) protein involved in leucine biosynthesis, and known to be phosphorylation controlled (Sekowska *et al*., 2004; Mishra *et al*., 2010, respectively) were also amplified by phosphorylation.

From the Tables 3.6 and 3.7, was also noticed that all the proteins affected by the short-term elicitation had a pI ranging between 4 – 6, therefore in future work for a better resolution of the proteins a narrow pH range (4-7) will be used.

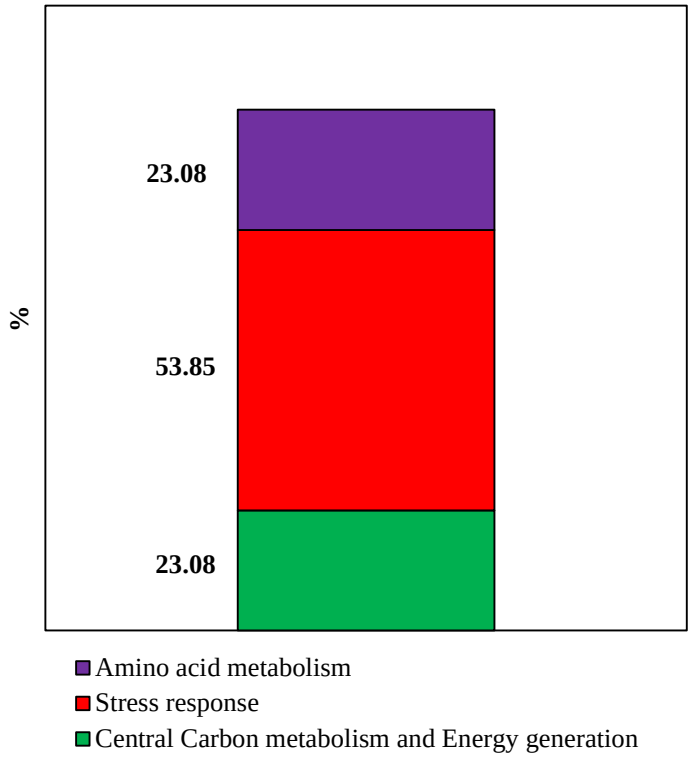


Figure 3.39 Functional grouping of the cytosolic *B. licheniformis* phosphoproteins affected by the short treatment of OG/MO.

3.6.1.2.2 Comparison of whole proteome and phosphoproteome of short-term elicited *B. licheniformis*

For most of proteins, the experimentally determined pI is generally very close to the one anticipated by software prediction. In other situations, where the pI is observed to be shifted from the expected value, it is shown that this shift is often correlated to protein modification, including PTM such as phosphorylation that can shift the proteins pI by several pH units.

Proteins extracted from *B. licheniformis* after short-term elicitation, were separated on 2-D gels and stained for both phosphoproteome and whole proteome (Figures 3.38 and 3.36, respectively). The two images were digitally coloured and overlaid (Figure 3.40) to determine the relative position of the phosphoproteins on the whole protein stained image. The figures shows that proteins 24 and 91 were shifted towards a more acidic pI, compared to the corresponding non-phosphorylated proteins 24 and 91. This is consistent with one or more

phosphorylation sites. However for the others identified phosphorylated proteins, the addition of the phosphoryl groups did not cause any shifts in their pI on the gels. It has been suggested that the effects of phosphorylation on pI shift can vary depending upon the proteins and the amount of phosphorylation. Moreover, large changes in the pI are often observed for proteins with a pI above 7.0 upon phosphorylation, whereas little change is observed for proteins with a pI of around 5 (Zhu et *al.*, 2005).

A comparison of the fold increase (as compared to the related control protein) of the phosphorylated and non-phosphorylated proteins obtained from the test gels is shown in Table 3.8. The normalised volume of the non-phosphorylated proteins; 2, 24, 102, 125 and 93 demonstrated to be equally affected by the presence of the elicitor in the cultures (values in red), since the levels of expression increased as compared to the control non-phosphorylated proteins. For the remaining spots, 27, 44, 54, 59, 62, 91 and 96, the non-phosphorylated proteins were less affected by the elicitors as compared to the phosphorylated peptides (black). This suggests that in these spots the protein activity was increased through phosphorylation without changing the protein's expression level.

Table 3.8 Comparison the fold increase (as compared to the related control spot) obtained in the phosphoproteome and whole proteome of *B. licheniformis* treated with OG and MO.

Fold increase as compared to the control spots		
Spot number	**Phosphorylated**	**Nonphosphorylated**
2	1.9	1.9
24	1.5	1.3
102	1.9	1.7
125	1.8	1.8
93	1.3	1.0
27	2.1	1.2
44	1.8	1.3
54	1.7	1.3
59	2.4	1.2
62	1.7	1.0
91	1.9	1.0
96	2.1	1.2

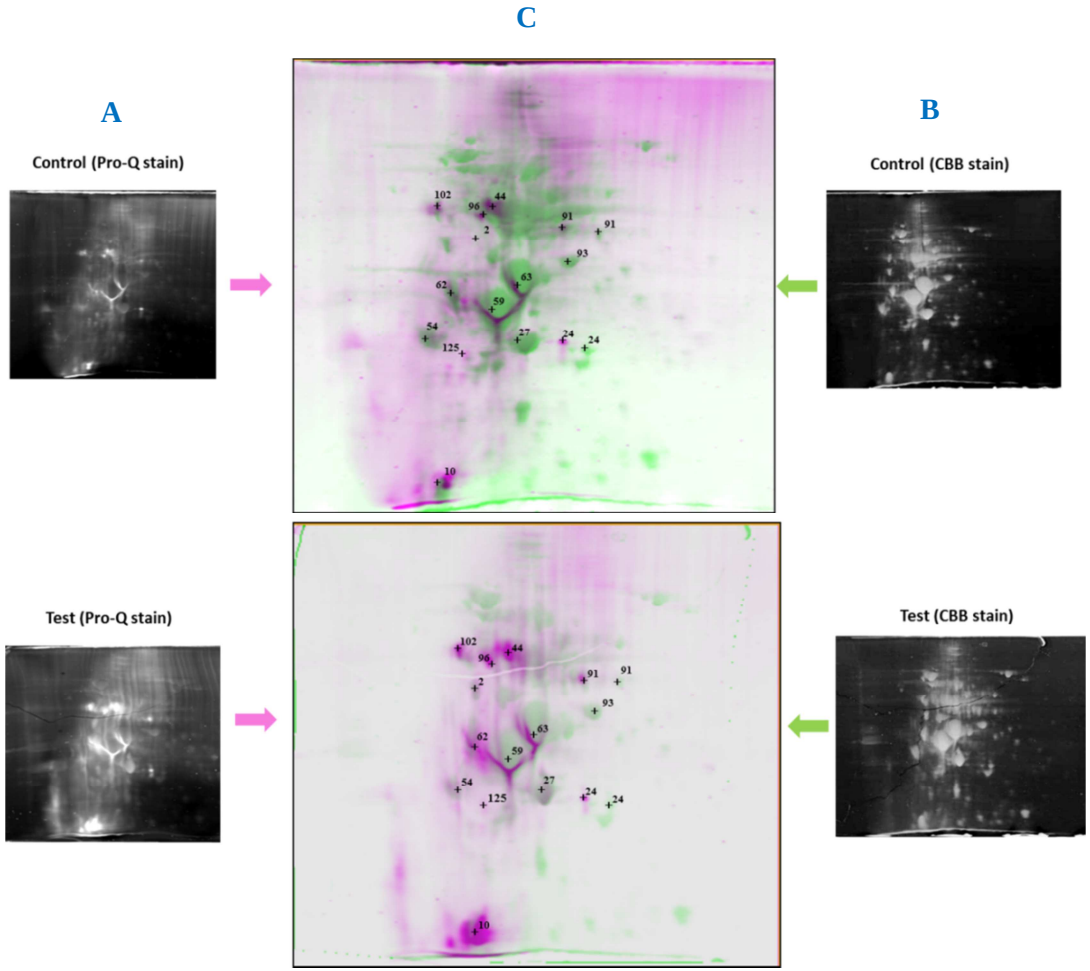


Figure 3.40 Phosphoproteome and whole proteome of *B. licheniformis* after short-term elicitation. Images generated by Progenesis PG240 Samespot software. **(A)** Pro-Q Diamond stained image of gels of control and test gels (pink) and **(B)** Image of the same gel stained with CBB stain (Coomassie Blue (green). **(C)** The 2 images overlaid on top of each other. Image C used to determine the relative position of the Pro-Q Diamond stained spots on the Coomassie stained image for spot comparison.

3.6.2 Proteomic profile of *B. licheniformis* supplemented cultures after long-term elicitation

3.6.2.1 Comparative pattern of whole proteome of *B. licheniformis* subjected to long-term elicitation

In order to further the knowledge on the effects caused by the elicitors during the microbial growth, we investigated the profile of proteins responding to long-term exposure to OG and MO by performing a comparative proteomic analysis of untreated and treated *B. licheniformis* cultures (Figure 3.41). Approximately 575 proteins were resolved between pH 4-7 of which 30 proteins from the test gels were significantly different when compared to the proteins from the control gels ($p \leq 0.05$, replicates = 6). Among these, 21 proteins (9 up-regulated and 12 down-regulated) were selected for identification by LC-MS/MS. This selection was based on the expression levels (fold change ≥ 1 or ≤ 1) and the position of the protein species in relation to spots; only spots that could be excised without risk of mixing were selected for identification.

The normalised volumes of selected proteins and their identities, obtained by LC-MS/MS are shown in Tables 3.9 and 3.10, respectively. MS/MS spectra were analysed on the Mascot database.

The results showed that in some cases, two or more spots differing in their pI or MW were found to be the same protein. For example, oxalate decarboxylate was detected in five spots (30, 32.1, 229, 240, 245 and 405) and translocation dependent antimicrobial spore component in three spots (269, 402 and 405). This suggests the presence of different isoforms of the same protein or that a possible post-translation modification (PTM), such as phosphorylation or glycosylation, has taken place on the protein.

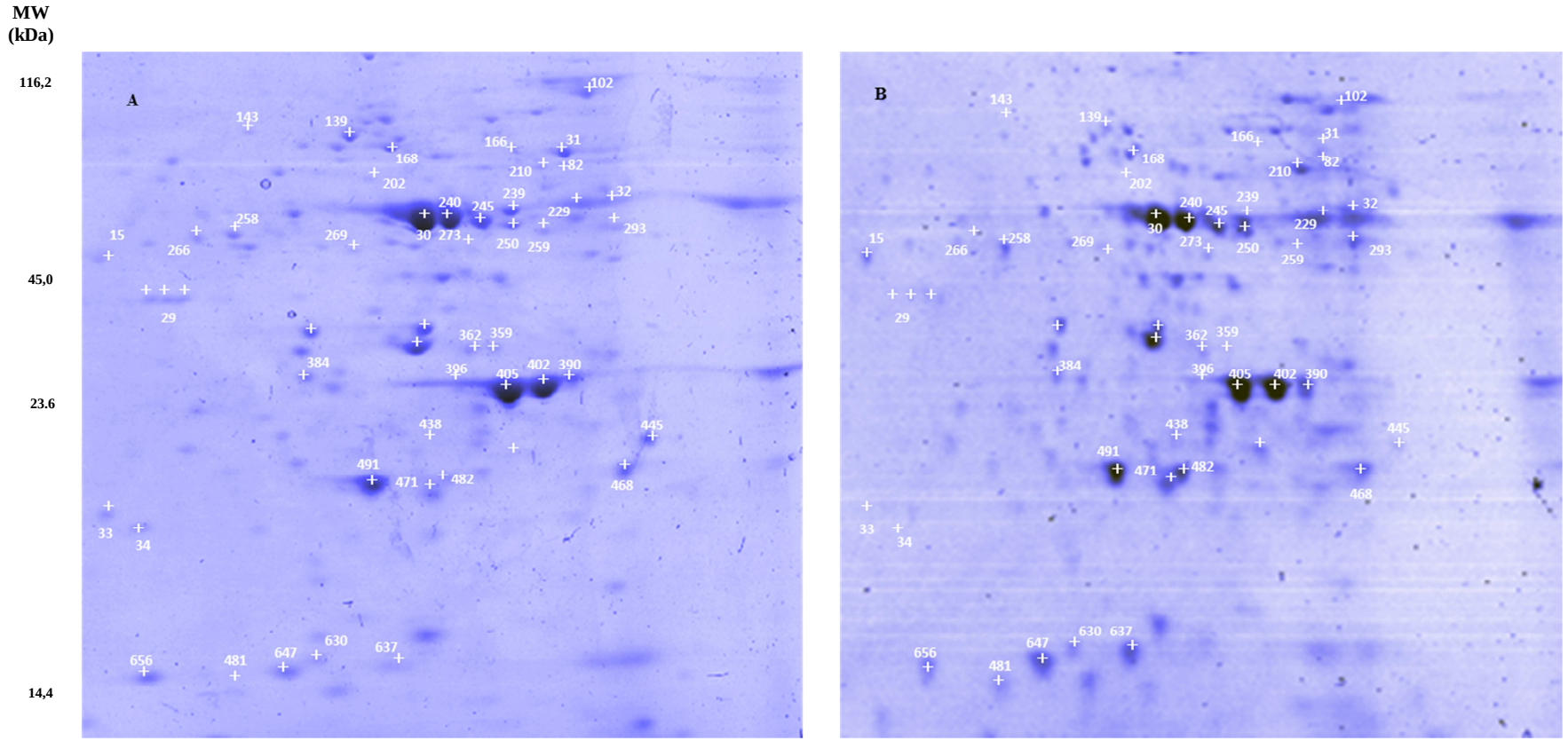


Figure 3.41 Comparative 2-DE gels showing the whole proteome of *B. licheniformis*, after long-term elicitation: (A): Control culture (no elicitor added) and (B) culture supplemented with OG at 0 hrs and MO after 24 hrs. Proteins were focussed on a pH 4-7 linear IPG strip (GE-Healthcare), separated by 2-D gel electrophoresis and stained with Coomassie Blue. Number represents the protein spots.

Table 3.9 Proteins of *B. licheniformis* after long-term elicitation with OG and MO. Anova analysis generated by Progenesis Same Spots software (Non-Linear Dynamics, UK). Fold increase obtained as compared to control spots.

Spot number (Refer to Figure 3.39)	Anova (*p*)	Fold change	Average Normalised Protein Volumes	
			Control	Test
Up-regulated – Fold change (≥ 1)				
30	0.042	1.5	1.932e+006	1.332e+006
239	0.043	2.5	3.047e+004	7.745e+004
250	0.042	2.4	3.047e+004	7.745e+004
240	0.037	1.7	1.209e+006	7.038e+005
245	0.027	1.9	4.148e+005	7.913e+005
293	0.006	1.7	5.185e+004	9.033e+004
384	0.034	1.5	1.274e+005	8.331e+004
405	0.022	2.3	1.352e+006	3.063e+006
438	0.010	1.7	2.000e+004	3.417e+004
Down-regulated – Fold change (≤ 1)				
31	0.037	0.63	2.594e+005	1.638e+005
32	0.032	0.75	4.137e+005	3.118e+005
139	0.042	0.68	9.061e+004	6.127e+004
229	0.039	0.85	3.902e+005	3.307e+005
258	0.005	0.46	8.716e+004	3.986e+004
259	0.006	0.58	8.144e+004	4.687e+004
266	0.040	0.74	4.456e+004	3.287e+004
269	0.038	0.71	2.871e+004	2.034e+004
402	0.039	0.56	1.941e+006	1.081e+006
362	0.018	0.75	1.747e+005	1.309e+005
468	0.033	0.55	3.606e+005	1.990e+005
491	0.028	0.61	3.496e+005	2.132e+005

Table 3.10 Long-term elicited proteins of *B. licheniformis*. Characteristics of the proteins separated by two-dimensional electrophoresis, detected by Coomassie Blue and identified by LC-MS/MS.

Spots number / putative protein	Acession number[a)]	Gene	Protein function	MW (kDa) / pI	Sequence coverage (%)	LC-MS/MS	
						Mascot Score	N° of peptides[b)]
Up-regulated – Fold increase (≥ 1)							
384 3-hydroxyisobutyrate dehydrogenase	52080476	*BL00292*	Valine degradation	32.2/5.0	14.9	111.8	2
438 Elongation factor G	15674452	*fus*	Translation	76.5/4.7	2.2	88.1	1
293 Elongation factor Tu	16077181	*tufA*	Translation	43.6/4.8	12.4	301.7	4
30 Oxalate decarboxylase	52081879	*oxdC*	Sporulation	43.5/5.0	8.1	218.5	3
240 Oxalate decarboxylase	52081879	*oxdC*	Sporulation	43.5/5.0	13.8	368.0	4
245 Oxalate decarboxylase	52081879	*oxdC*	Sporulation	43.5/5.0	12.0	302.7	3
405 Translocation dependent antimicrobial spore component	52080998	*tasA*	Sporulation	28.8/5.3	14.4	282.6	4
239 Isocitrate dehydrogenase	52786790	*icd*	Citric acid cycle	48.0/4.9	6.8	116.6	3
250 Isocitrate dehydrogenase	52786790	*icd*	Citric acid cycle	48.0/4.9	6.4	116.6	3
Down-regulated – Fold increase (≤ 1)							
266 Threonine synthase	52081719	*thrC*	Threonine biosynthesis	37.6/5.5	3.7	54.7	1

258 Ketol acid reductoisomerase	16079881	*ilvC*	Valine, leucine biosynthesis	37.4/5.4	5.4	153.1	2
2.3 Chaperonin GroEL	52079060	*groEL*	Protein folding	57.5/4.6	3.5	79.0	1
31 ATP synthase	56421893	*GK3358*	Energy generation	51.8/4.9	8.5	213.2	3
259 Glycerol dehydrogenase	52079262	*gldA*	Glycolysis	39.5/4.6	11.4	203.7	3
139 Aldehyde dehydrogenase	52785994	*dhaS*	Glycolysis	54.4/5.2	7.8	169.4	2
269 Translocation dependent antimicrobial spore component	52080998	*tasA*	Sporulation	28.8/5.3	8.7	117.4	2
402 Translocation dependent antimicrobial spore component	52080998	*tasA*	Sporulation	28.8/5.3	25.0	477.7	6
32.1 Oxalate decarboxylate	52081879	*oxdC*	Sporulation	43.5/5.0	12.0	251.0	3
229 Oxalate decarboxylase	52081879	*oxdC*	Sporulation	43.5/5.0	8.1	170.5	3
468 Alkyl hydroperoxide reductase	52080021	*ykuU*	Oxidative stress	20.4/4.4	15.0	211.3	2
491 Superoxide dismutase	52081038	*sodA*	Oxidative stress	22.5/5.2	27.7	389.1	5

a) Accession numbering (entries in NCBInr).
b) The sequenced peptides identified by LC-MS/MS are indicated below:
Spot 30: K.IPHNVER.D, K.FSFSDVHNR.L, K.TIASALVEVEPGGIR.E
Spot 31: R.FTQAGSEVSALLGR.M, R.ALAPEIVGEEHYQVAR.K, K.YDHLPEDAFR.L
Spot 32: K.FSFSDVHNR.L, K.TIASALVEVEPGGIR.E, K.DDHYADVSLNQWLALVPEELVR.Q
Spot 139: K.ITGQTIPVNGPYFNYTR.H, K.EEIFGPVLAAIPYETVDEVIER.A
Spot 229: K.IPHNVER.D, K.FSFSDVHNR.L, K.TIASALVEVEPGGIR.E
Spot 239: R.AAIEYAIEHGR.K, K.VFTWAEYDR.I, K.VVTYDFAR.L
Spot 240: K.IPHNVER.D, K.FSFSDVHNR.L, K.TIASALVEVEPGGIR.E, K.DDHYADVSLNQWLALVPEELVR.Q
Spot 245: K.FSFSDVHNR.L, K.TIASALVEVEPGGIR.E, K.DDHYADVSLNQWLALVPEELVR.Q
Spot 258: R.YSISDTAQWGDFVSGPR.V, K.EWIVENQVNRPR.F
Spot 259: K.FNYECTQEEIDR.N, R.FFAAGIGDALATYFEAR.A, K.DLGVDEFVEEEWR.Q
Spot 266: K.EPIALVNSVNPYR.I
Spot 293: R.GITISTAHVEYETETR.H, K.IFELMDAVDEYIPTPER.D, K.IFELMDAVDEYIPTPER.D, K.LLDYAEAGDNIGALLR.G
Spot 269: K.AIEPEFLHDNGK.I, K.VQLVIEFVNDK.T
Spot 384: K.GLVHLAEPGVLLIDTSTVAPELNIR.I, K.GIDFLAAPVSGGVIGAVNR.T
Spot 402: K.EVLMQVGYSNFVDGNAK.N, K.EVLMQVGYSNFVDGNAK.N, K.STAEDFLK.Q, K.NIILDEANLYDLYNMSAK.K, K.AIEPEFLHDNGK.I, K.VQLVIEFVNDK.T
Spot 405: K.STAEDFLK.Q, K.AIEPEFLHDNGK.I, K.DPYDFDK.V, K.VQLVIEFVNDK.T
Spot 438: K.GFEFENAIVGGVVPR.E,
Spot 468: K.YPLAADTNHEVSR.E, R.EYGVLIEEEGVALR.G
Spot 491: K.HHNTYVTK.L, K.SVEELVANLDAVPENIR.T, K.FGSFDQFK.E, K.EDFAAAAAGR.F, K.AFWNVVNWDEVAR.L

3.6.2.2 Summary of long-term elicited proteins of *B. licheniformis*

A summary of the functional grouping of the cytosolic *B. licheniformis* proteins affected by the long treatment of OG/MO are shown in Figure 3.42. The significance of these findings will be further discussed in Chapter 4.

Although the glycolytic enzymes, glycerol dehydrogenase (species 259), aldehyde dehydrogenase (139) were down-regulated, two isoforms of isocitrate dehydrogenase (species 239 and 250), a major regulatory point in the TCA cycle, and a protein involved (384) in amino acid degradation were up-regulated. This suggests that the carbohydrate metabolism of *B. licheniformis* might be partially impaired when this organism is exposed to long-term elicitation. This implies that *B. licheniformis* cells obtain energy, at least at some extend, via TCA through the recycle of non-required cellular components such as amino acid.

Despite the fact that two elongation factors G and Tu (species 438; 293) were up-regulated, two proteins involved in amino acid biosynthesis (species 258; 266), one involved in protein folding (species 2.3) and one protein involved in energy

generation (ATP synthase) were down-regulated. This can be due to the down-regulation of the ATP synthase, which suggests that cellular energy status plays a role in shutting down protein synthesis under long-term elicitation of OG and MO. The presence of the OG and MO also triggered the down-regulation of stress proteins such as SOD, Alkyl hydroperoxide reductase, oxalate decarboxylase and translocation dependent antimicrobial spore component.

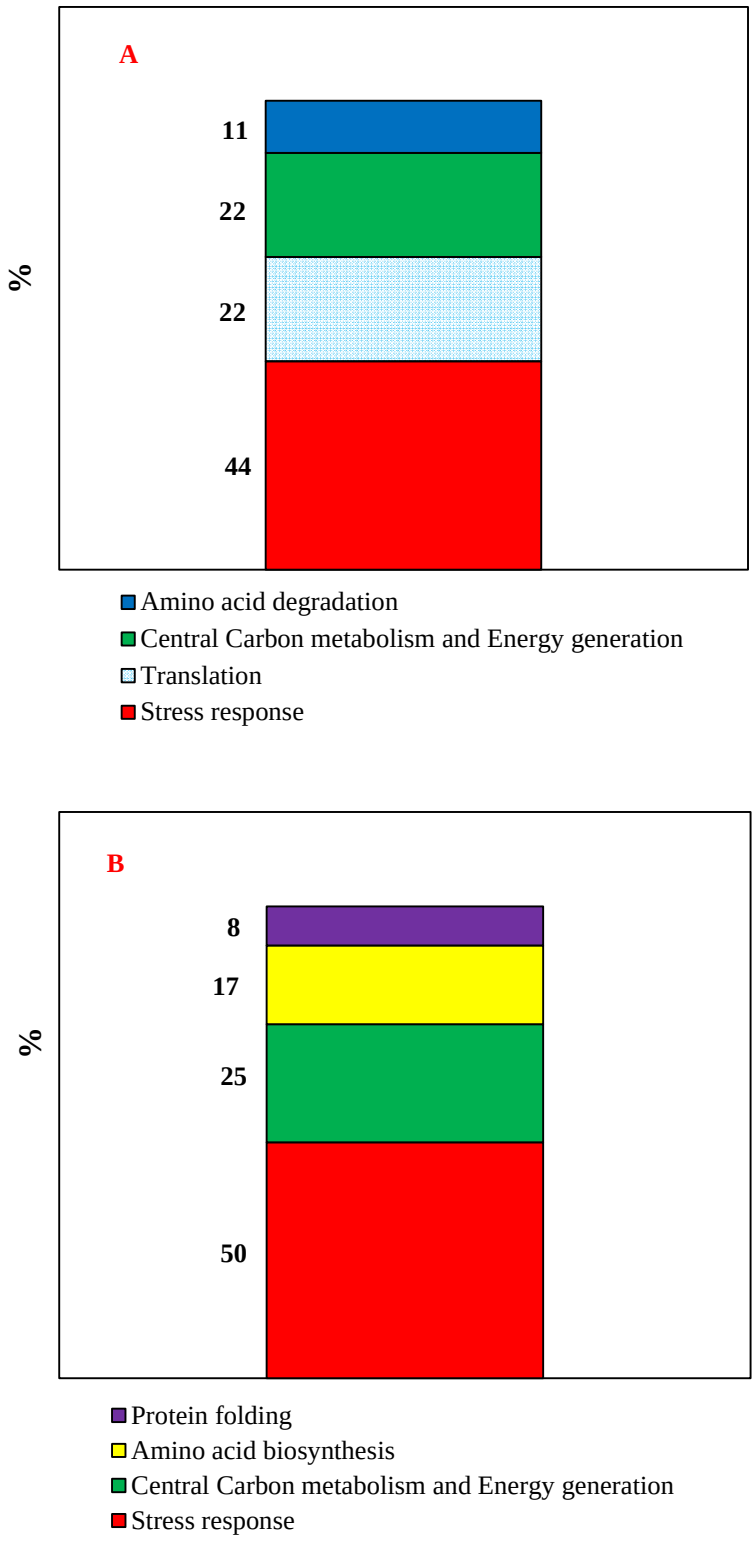


Figure 3.42 Functional grouping of the cytosolic *B. licheniformis* proteins affected by the long treatment of OG/MO. (A) up-regulated proteins and (B) down-regulated proteins.

3.6.3 Reactive oxygen species (ROS) generation, SOD and catalase activity

Previously we reported changes in expression and phosphorylation of proteins involved in ROS defence mechanism by the presence of OG and MO in cultures of *B. licheniformis* (section 3.6.2.2). Whilst levels of these proteins were reduced in terms of expression, their phosphorylation intensity was significantly increased by the short-term elicitation. As phosphorylation of proteins is a known response element in microbial stress (Hecker *et al*., 2007), able to increase activity of enzymes, we decided to analyse changes in ROS levels, SOD and catalase activity in response to the addition of OG and MO to the *B. licheniformis* cultures during the first 30 min post MO addition.

3.6.3.1 Generation of ROS

The addition of elicitors has been reported to affect reactive oxygen species (ROS) in fungi and filamentous bacteria (Radman *et al*., 2006; Sangworachat, 2006). The effect of long-term elicitation (24, 32, 48, 52, 60 and 72 hrs) on ROS levels was also reported in *B. licheniformis* (Murphy, 2008). The authors reported a reduced ROS levels concomitant with an increased catalase activity. However, such an effect has not been described during short sampling intervals (min).

A comparison of ROS levels in *B. licheniformis* culture treated with the combined OG and MO is shown in Figure 3.43. In the test culture, an increase in ROS profile was observed as early as 5 min after MO addition and the increased pattern was maintained through the course of the experiment ($p \leq 0.01$). The highest increase of approximately 171 % in ROS production was seen after 5 min, followed by 112 % after 10 min of stimulation with OG and MO. Although decrease in ROS was showed during a long-term elicitation (Murphy, 2008), this study shows that the presence of OG and MO are able to alter ROS levels considerably during a short-term, suggesting that the perhaps the cell's response to the elicitor might be associate with the physiological state of the cells.

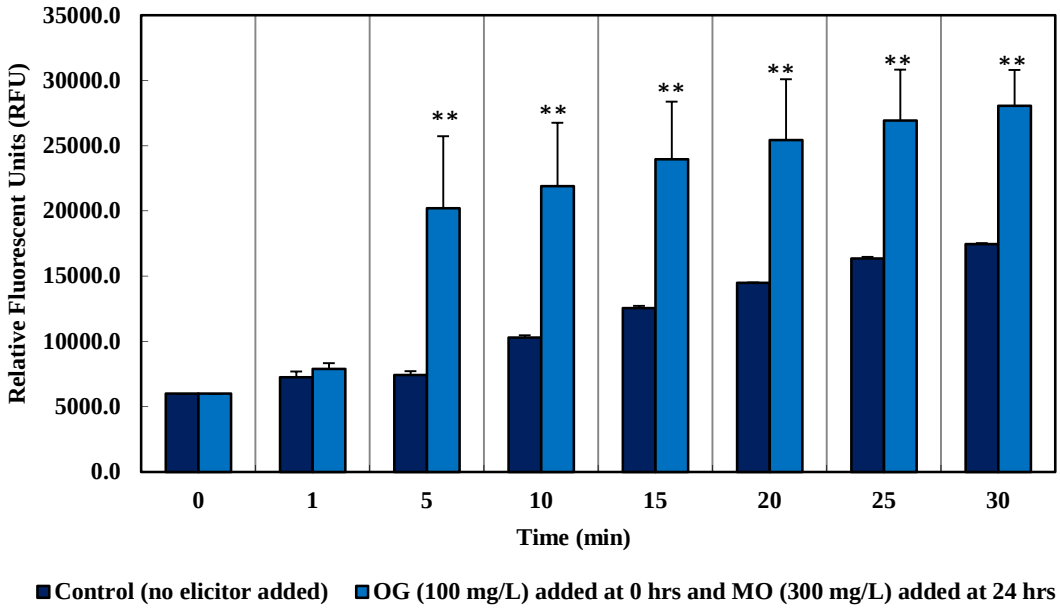


Figure 3.43 Effect of oligosaccharide elicitors on the ROS profile of *B. licheniformis* cultures. Control (no elicitor added) and Test culture: OG (100 mg L^{-1}) added at 0 hrs and MO (300 mg L^{-1}) added at 24 hrs. Experiments were carried out in triplicate. Error bars indicate standard deviation between triplicate samples.

3.6.3.2 Effect of elicitors on superoxide dismutase (SOD)

In this study the effect of OG and MO added at 0 and 24 hrs, respectively, on intracellular SOD was investigated. Samples were taken from *B. licheniformis* culture at 5, 10, 20 and 30 min after the MO addition. Cells were then disrupted and SOD activity was measured (Figure 3.44). Highest SOD activity were ($p \leq 0.05$) detected in test culture after 5 min (110 % increase as compared to the control culture), followed by 30 min (109 % increase as compared to the control culture) post-MO stimulation of *B. licheniformis* culture. Although still significantly higher than the control samples, a decline in SOD activity were observed in test samples taken after 10 and 20 min.

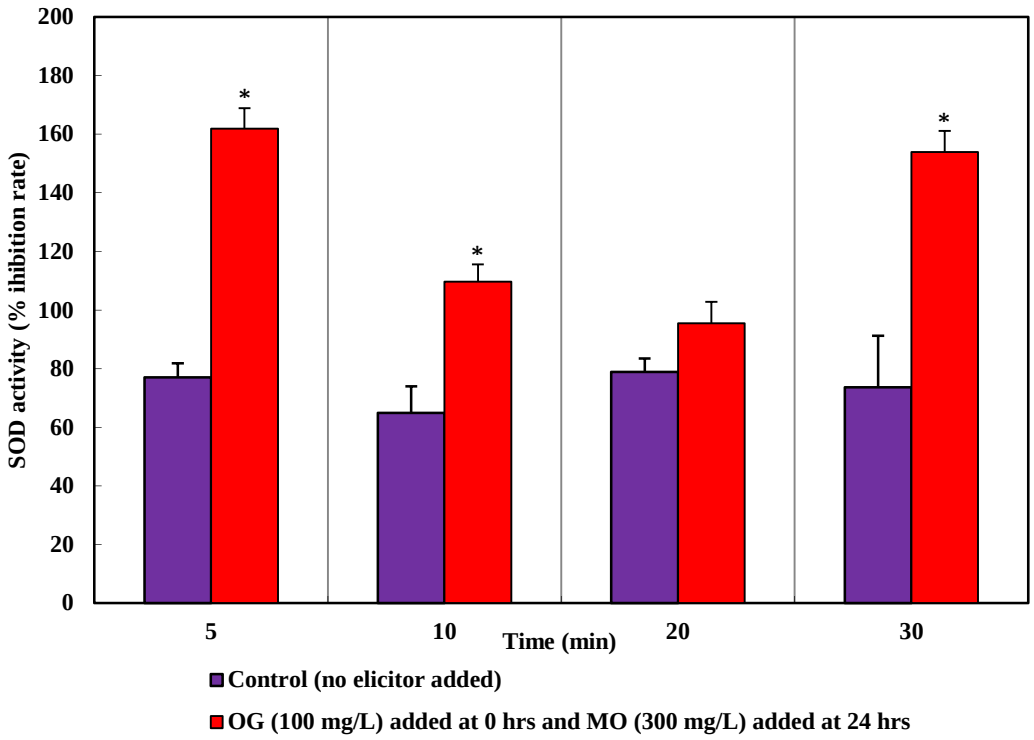


Figure 3.44 Effect of oligosaccharide elicitors on SOD production of *B. licheniformis* cultures. Control (no elicitor added), and Test culture (OG/MO: 100/300 mg L^{-1}) added at 0 hrs and/or 24 hrs. Experiments were carried out in triplicate. Error bars indicate standard deviation between triplicate samples.

3.6.3.3 Catalase activity

Addition of elicitors has been reported to affect catalase activity in filamentous bacteria (Sangworachat, 2006). Long-term elicitation (24, 32, 48, 52, 60 and 72 hrs) was also reported to increase catalase profile of *B. licheniformis* (Murphy, 2008). However, such an effect has not been reported during short sampling intervals (min).

In this study the effect of OG and MO elicitor added at 0 hrs and 24 hrs, respectively, on intracellular catalase was investigated. Higher catalase activity was detected during the course of the experiment for the control culture (Figure 3.45). Maximum catalase activity was 0.04 μmol min^{-1} mL^{-1} for control samples taken after 5 min. Although significantly higher than the test culture, catalase activity from control samples declined after 5 min. This result suggests that in this elicited system catalase is not the primary enzyme applied to scavenge H_2O_2 generated after the reaction of SOD and O^-_2. Alkyl hydroperoxide dismutase (Ahp) has been reported to be a more efficient scavenger of trace H_2O_2 than catalase (Seaver and Imlay, 2001). As the phosphorylation state of Ahp was showed to be increased by the presence of OG and MO (Section 3.6.2.2), it is believed that Ahp activity was highly to be increased as well. Unfortunately this study has not found a suitable methodology to measure Ahp. Therefore, further investigation of the role of Ahp as response to the elicitors is required.

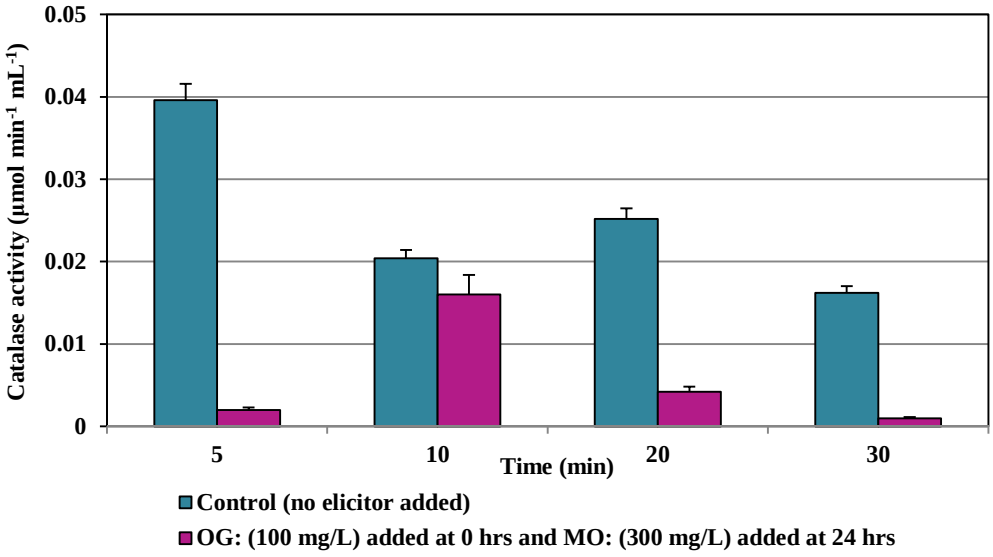


Figure 3.45 Effect of oligosaccharide elicitors on catalase production of *B. licheniformis* cultures. Control (no elicitor added), and Test culture (OG/MO: 100/300 mg L^{-1}) added at 0 hrs and/or 24 hrs. Error bars indicate standard deviation between triplicate samples.

Chapter 4

4.0 Discussion

4.1 Bacitracin A production

In this work, the effect of oligosaccharide elicitors on the bacitracin A production in cultures of *B. licheniformis* was investigated. Highest bacitracin A production was observed when stationary growth phase was reached. The production subsequently declined, as reported by other investigators (Haavik, 1974b; Murphy, 2008). This decline is associated with the increased pH, observed throughout the course of fermentation. The stability of bacitracin A is well known to be pH dependent. It has been established that under alkaline conditions (above pH 8) bacitracin A is converted to bacitracin F, via oxidative deamination of thiazoline ring to ketothiazole (Konisberg *et al*., 1961). On an industrial level, the conversion of bacitracin A to the unwanted bacitracin F is avoided by pH controlled fermentation (Hickey, 1964).

In this study, levels of bacitracin A obtained in SF were 16 % higher than those obtained from STR. This difference was thought to be related to differences in cell growth rate. The maximum specific growth rate (μ) and doubling time (T_d) were determined to be $\mu = 0.24\ h^{-1}$, $T_d = 2.79$ hrs for SF control culture and $\mu = 0.28\ hrs^{-1}$, $T_d = 2.43$ hrs for STR control culture. Better conditions (i.e. aeration and mixing) were provided in the bioreactor, which led to higher growth rates, compared to SF. Growth rates have been reported to be dependent on the dissolved oxygen (% DOT) where higher oxygen transfer or agitation rate leads to higher consumption of nutrients (Calik *et al*., 1998).

Although higher specific growth rates were observed in STR cultures, lower levels of bacitracin A were obtained compared to SF fermentation (Figure 4.1), this could be due to the rapid increase in culture pH promoted by higher growth rates in STR. Similar observations were made by Haavik (1974a; 1974b) and Murphy, (2008), who reported that bacitracin A production is negatively affected by increased pH.

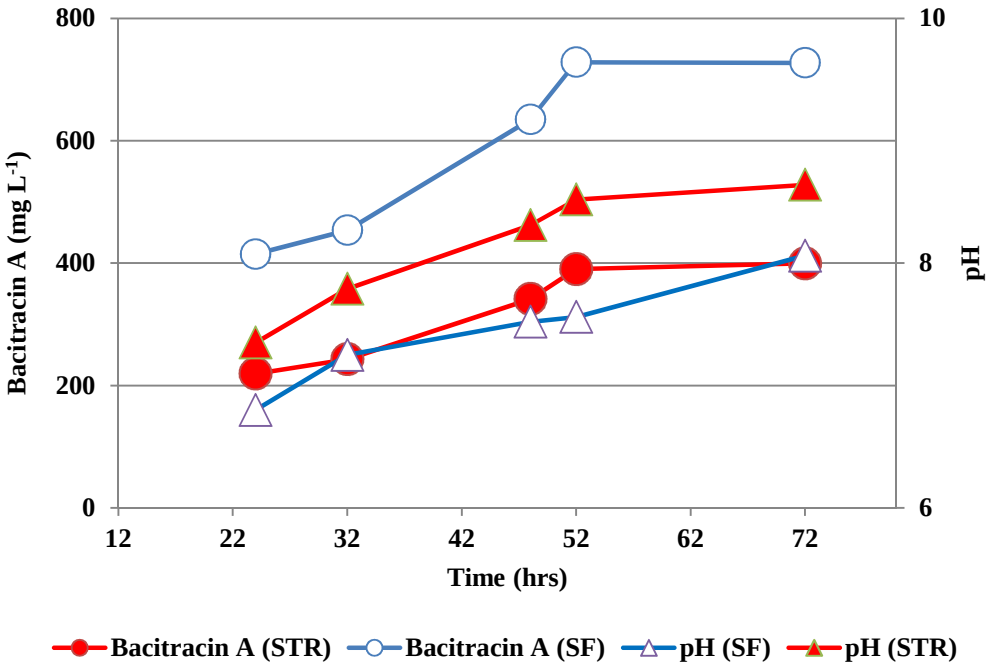


Figure 4.1 Bacitracin A production and pH profiles in shaken flasks and stirred tank reactors.

4.2 Effect of elicitors' concentration on *B. licheniformis* cultures

Despite the fact that previous elicitation studies revealed that the concentration of OG, OM and MO elicitors is an important factor for the enhanced production of a wide range of microbial metabolites (Ariyo *et al*., 1997; Sangworachat, 2006; Nair, 2007) including bacitracin A (Murphy, 2008), there has been no systematic investigation of the effective minimal and maximal concentrations of these elicitors in microbial cultures.

Lower doses (25 to 50 mg L^{-1}) of OG, OM and MO had no effect on growth of *B. licheniformis* and bacitracin A production. Conversely, higher concentrations of the same elicitors (500 to 100 mg L^{-1}) resulted in overproduction of bacitracin A concomitant with increased biomass production. These clearly suggest that the effects generated by these elicitors on *B. licheniformis* cultures are dose-dependent above a certain concentration.

Elicitation studies carried out in plant cell cultures, showed that the use of low doses of a deacetylated chitooligosaccharide elicitor caused no effect on phenylalanine ammonia-lyase and H_2O_2 production (Cabrera *et al*., 2006). They suggested that the deacetylated chitooligosaccharide loses its biological activity as an elicitor in *Arabidopsis* cultures, when concentrations lower than 300 mg ml^{-1} are used. Akimoto *et al*., (1999) reported that the minimum elicitor concentration for elicitation to begin is an indication of the sensitivity of the cells in recognising the elicitors. According to Murphy, (2008) the *B. licheniformis* defence mechanism to the elicitor might occur through a signalling event caused by the binding of the elicitor to cell membrane receptors (lectins), which leads to the activation of genes involved in bacitracin A production. Hence, it could be speculated that at a very low concentration, these oligosaccharides lose their effectiveness as elicitors, probably due to low binding efficiency to the elicitor-cell receptor.

Lectins are sugar binding proteins present on the bacterial cell surface acting in cell to cell recognition (Imberty *et al*., 2005). They are reported to have a function in protection against infectious agents in prokaryotes (Sharon, 2006). Lectins have been isolated from *Bacillus* spp. (Cole et *al*., 1984) including *B. subtillis* (Lakhtin *et al*., 1993; Kishko *et al*., 1997 and 1998). In *B. subtillis* the isolated lectin was demonstrated to be highly specific for N-glycolylneuraminic acid, N-acetylneuraminic acid and fructose 1.6-biphosphate (Lakhtin *et al*., 1993). Lectins isolated from *B. polymyxa 1460* (Karpunina *et al*., 2003) and *B. thuringiensis* (Akao *et al*., 2001), have been found to have great specificity for glucuronic acids and galactose, respectively. In *E. coli*, lectins with mannose and galabiose specificity were isolated, further suggesting that individual bacteria might express more than one lectin (Firon *et al*., 1984). To date, there is no lectin that has been identified in *B. licheniformis.* However, considering the organisational similarities between the *B. licheniformis* and *B. subtilis* genomes (Rey *et al*., 2004) it is highly likely that some lectins exist in this system. Nevertheless, further investigation is required to prove this.

The addition of higher doses of elicitors to *B. licheniformis* cultures caused overproduction of bacitracin A concomitant with increased biomass. In this study, a compound is considered an elicitor when its addition to living system increases metabolite production without affecting its normal growth. Therefore, at higher doses, OG, OM and MO are no longer classified as elicitors; even though Rhee and co-workers (2010), considered yeast extract as an elicitor, in spite of the fact that the improved production of decursinol angelate, a root specific secondary metabolite in cultures of *Angelica gigas,* occurred together with increased biomass.

4.3 MO composition and detection in liquid cultures of *B. licheniformis*

Detection of MO in liquid cultures of *B. licheniformis* using a phenol sulphuric acid assay was masked due to the production of carbohydrates by the bacterium. This observation was in line with a previous study (Murphy, 2008). For this reason, samples taken from supplemented cultures of *B. licheniformis* (SF and STR) were analysed by HPAEC and/or TLC chromatography for MO detection, during growth of the bacterium.

All the results obtained from shaken flasks (Sections 3.2) and bioreactors (Section 3.4) suggested that MO was gradually broken down by *B. licheniformis* over the course of the fermentation, with mannose being the end product of the degradation. Moreover, the results also suggested that this degradation was catalysed by the enzyme ß-mannanase, induced within 3 minutes of MO addition to the culture (between sampling and processing). ß-mannanase has been widely reported to be produced by *Bacillus* species, including *B. licheniformis* in the presence of mannan oligosaccharides, glucomannans and galactomannans (Feng *et al*., 2003; Wang *et al*., 2005; Songsiriritthigul *et al*., 2010). Furthermore, it was interesting to see that MO was able to induce β-mannanase within such short periods of time. Measurements of the kinetic induction of several enzymes of *E. coli*, have shown that for each enzyme a lag of about 3 mins between addition of inducers and appearance of enzymes exists (Pardee and Prestige, 1961). Similar results were obtained during measurement of ß-galactosidase induction in *E. coli* (Boezi and Cowie, 1961). They reported a lag of about 2.5 to 3 minutes after the

inducer addition and enzyme appearance. Similar results were shown elsewhere (Kepes, 1963; Muller-Hill *et al*., 1964; Scheleif *et al*., 1973; Dickson and Markin, 1980).

Interestingly, ß-mannanase was also expressed in the control culture (without elicitor supplementation) at the later stationary phase of the *B. licheniformis* growth. This observation suggests that the carbohydrates produced by this bacterium this period, might be inducing the enzyme production. ß-mannanase derived from *Bacillus* spp. is an inducible enzyme which displays tight specificity towards mannan oligosaccharides, galactomannas and glucomannas substrates (Tailford *et al*., 2009). Although the production of carbohydrates (Guerrini *et al*., 1998; Sutherland, 2001) as well as the characterisation of a varied range of polysaccharides produced by *Bacillus* spp., including *B. licheniformis* (Ramanathan *et al*., 2011) have been described, to our knowledge there are no reports stating specific production of the carbohydrates inducers of ß-mannanase in *B. licheniformis* cultures. Further studies are required to identify the carbohydrate components secreted by this bacterium.

4.4 Effect of the MO components (mannose, mannobiose and mannohexaose) on bacitracin production

The addition of MO (300 mg L^{-1}) to cultures of *B. licheniformis* is known to result in enhanced bacitracin A production (Murphy, 2008). As mannose, mannobiose and mannohexaose were established as the main constituents of the MO elicitor (Figures 3.8), it was important to investigate the role of these saccharides independently on the enhanced bacitracin A production of MO in *B. licheniformis* culture. Concentrations used in this study were based on the MO constituents in 300 mg L^{-1}, mannobiose (136 mg L^{-1}), mannose (121 mg L^{-1}) and mannohexaose (26.5 mg L^{-1}).

Addition of mannose, mannobiose and mannohexaose to *B. licheniformis* cultures generated diverse effects on the levels of bacitracin A production. The addition of mannobiose produced the highest and most sustained increase in bacitracin A concentration whereas in mannose and mannohexaose treated culture the effects were less (Figure 3.15). This implies that mannobiose exhibits higher biological

activity than mannose and mannohexaose. As similar levels of bacitracin A were obtained in MO and mannobiose treated cultures, and as the presence of mannobiose is seen to be constant during the bacterium growth, it is plausible to say that mannobiose is the main biologically functional unit capable of affecting production of bacitracin A in *B. licheniformis* cultures.

TLC and HPAEC results of samples taken from *B.licheniformis* treated cultures showed that mannobiose and mannohexaose were gradually degraded by ß-mannanase into mannose, which in turn, disappeared with time. The disappearance of mannose could be due to its consumption by the bacterium. It is known that *B. licheniformis* is capable of utilising various sugars; including mannose (Durand *et al*., 1979; Nilegaonkar *et al*., 1996; Van Dyk, 2009) for the production of bacitracin A (Qadeer *et al*., 1988). A study involving characterisation of the cellulolytic system of *B. licheniformis* showed the ability of this bacterium to utilise mannose to the same extent as glucose (Van Dyk, 2009). The catabolic pathway for mannose has been reported in *Bacillus* spp. (Saier, 1977; Sun and Altenbuchner, 2010) including in *B. licheniformis* (Stülke and Hillen, 2000). In bacterial systems, extracellular mannose is taken up by the cells and directed into the glycolytic pathway by the phosphoenolpyruvate: carbohydrate phosphotransferase transport system (PTS) (Saier, 1978; Postma *et al*., 1993).

Kremling *et al*., (2007) proposed that the PTS regulation network not only controls carbohydrate uptake and metabolism but also acts as a sensory system. The authors suggest that sensor elements of PTS can also process external stimuli into intracellular signals. The occurrence of proteins capable of sensing and responding to the presence of mannobiose, phosphotransferase system enzyme II (PSEII), (Kunst *et al*., 1997) in *B. subtillis* genome is known. Therefore, given the organisational similarities between the *B.licheniformis* and *B. subtilis* genomes (Rey *et al*., 2004), we suggest that, in addition to lectins, the PTS sensory system might be an intermediary in the signalling events brought about by the presence of mannobiose in the liquid culture. Further investigation is required to prove this.

Overall, in my view, the enhanced-bacitracin A production observed in *B.licheniformis* as a result of MO addition is a process occurring by a range of cumulative events, which include (Figure 4.2): **1)** recognition of MO by the cell's receptor/sensor; **2)** Induction of ß-mannanase enzyme **3)** MO degradation by ß-mannanase; **4)** the degradation products of MO contribute to intracellular changes in different ways: mannose, mannobiose and mannohexaose interact with their putative cell sensor/receptor and activate the defence response mechanism (where mannobiose is found to be more effective than mannose and mannohexaose) **5)** after the interaction with the putative cell sensor/receptor; mannobiose and mannohexaose are further degraded into mannose, which in turn is probably taken up by the cell and incorporated into the energy generation pathways.

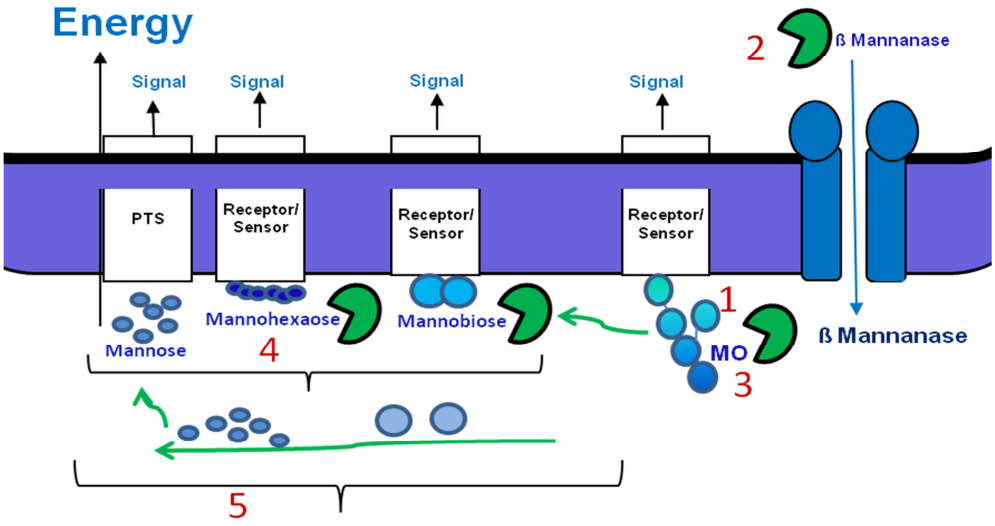


Figure 4.2 MO degradation products and their possible role during elicitation. **1)** recognition of MO by the cell's receptor/sensor; **2)** Induction of ß-mannanase enzyme **3)** MO degradation by ß-mannanase; **4)** the degradation products of MO contribute to intracellular changes in different ways: mannose, mannobiose and mannohexaose interact with their putative cell sensor/receptor and activate the defence response mechanism (where mannobiose is found to be more effective than mannose and mannohexaose) **5)** after the interaction with the putative cell sensor/receptor; mannobiose and mannohexaose are further degraded into mannose, which in turn is probably taken up by the cell and incorporated into the energy generation pathways.

4.5 Elicitation studies in Continuous culture

In batch fermentation, the addition of MO (300 mg L^{-1}) in cultures of *B. licheniformis* was reported to result in higher bacitracin A production (Murphy, 2008). In order to investigate if continuous MO stimulation would further increase the bacitracin A levels, a chemostat culture of *B. licheniformis* was set at 24 hrs (late exponential phase). ß-mannanase production and MO degradation during the continuous culture were also monitored.

In this experiment, the addition of MO to the chemostat culture of *B. licheniformis* caused no significant difference ($p \geq 0.05$) in bacitracin A production as compared to the control culture. This could be due to the physiological state of the *B. licheniformis* growth at which the continuous culture was set. In SF and STR batch cultures, initial changes in bacitracin levels were only observed during the

stationary phase of growth (8 hrs after MO was added in the late exponential phase of the *B. licheniformis* batch growth - Figure 3.21). Consequently, this provides evidence that the physiological stage of growth plays an indispensable role in elicitor-enhanced production of bacitracin A (Figures 3.26).

Although ß-mannanase activity was detected during the continuous culture, no MO degradation was observed (Figure 3.29). The changes observed in bacitracin A production, ß-mannanase activity and MO degradation in response to the elicitor (s), is most likely to be related to the changes in *B. licheniformis* cells throughout their life cycle; which could then affect the response of the cultures when the elicitor binds to its cell surface sensor/receptors. Marero and co-workers (1997) studying elicitation of indirubin production by *Polygonum tinctorium* using elicitors prepared from fungal cell walls of *Aspergillus niger, Rhizopus oryzae* and *Rhizoctonia solani,* proposed that the cells need to reach a specific physiological state before they can react to the stimulus and that addition of elicitor outside the specific physiological state could inhibit or have no effect on the treated cells.

Hence, I would suggest that a specific physiological state of growth needs to be reached before the *B. licheniformis* cells can perceive the MO stimulation and generate a response to it. This cell state-dependency might explain MO disappearance on TLC plates (Figure 3.29), the enhanced production of bacitracin A and ß-mannanase activity after the continuous culture was stopped and a batch culture re-established. It is important to highlight that unlike the continuous culture where the same physiological state is maintained during the process, the batch fermentation involves a microbial population undergoing continuous change.

Therefore we would propose that the overproduction of bacitracin A is not only as a result of the MO addition to the culture of *B. licheniformis,* but it is the outcome of the interaction with MO and the changes in cell physiology occurring during bacterial growth.

4.6 Metabolic response of *B. licheniformis* to oligosaccharide elicitors

4.6.1 Measurement of intracellular Ca^{2+} levels

Ca^{+2} as a universal messenger, transmits signals from the cell surface into the cytoplasm. Through temporary changes in the intracellular Ca^{2+}, cells respond to a number of stimuli (Dominguez, 2004). It has been reported that bacterial cultures are able to sense an external stimulus and respond to it via Ca^{2+} transients (Campbell, 1983). In bacterial cultures, changes in the cytosolic calcium levels trigger cell activation through protein phosphorylation (Norris *et al*., 1996).

In this work, the effect of oligosaccharide elicitors on the intracellular Ca^{2+} levels was investigated in *B. licheniformis*. Changes in Ca^{2+} influx were observed upon OM, OG and MO addition to the bacterial culture. A similar observation was made in *E. coli* and *B. subtilis* culture where increased cytosolic Ca^{2+} levels were obtained by the addition of OG and MO (Murphy *et al*., 2011).

Knight, (2000), cited that Ca^{2+} must play a role in relaying external stimuli and regulating reactions which lead to a new situation. Evidence for the role of Ca^{2+} as a secondary messenger during abiotic stress was obtained by measuring the Ca^{2+} dependency of a number of responses. Proline accumulation is a common physiological outcome of a wide range of abiotic and biotic stress (Verbruggen and Hermans, 2008). Parre *et al*., (2007) suggests that Ca^{2+}, as a secondary messenger, is involved in the proline accumulation shown by Arabidopsis cells under hyperosmotic stress. Cold shock stress induces nuclear cytoplasmic Ca^{2+} transients and increases the expression of a defence stress gene in tabacco cultures (Van der Luit *et al*., 1999). Moreover, in parsley cells, the pathogen defence reactions are induced by the elicitor only if Ca^{2+} is present in the medium (Nurnberger *et al*., 1994). In tobacco, soybean, carrot and parsley cultures, this requirement correlates with a rapid elicitor-stimulated Ca^{2+} influx, (Bach *et al*., 1993; Tavernier *et al*., 1995) suggesting that Ca^{2+} is an important element during elicitor mediated signal transduction, as it is in many other signalling processes.

Following the initial rise, Ca^{+2} levels dropped in all the test cultures as compared to the control culture. Murphy *et al*., (2011) studying the effect of these same elicitors on the intracellular Ca^{+2} influx in *B. subtilis* and *E. coli* cultures, showed that after the stimulus, levels of Ca^{+2} were re-established in all the test cultures. We propose that OG, OM and MO may cause a different response in Ca^{2+} transients in *B. licheniformis* as compared to *B. subtilis* and *E. coli*, and that these changes might be part of the cellular control mechanism used by the bacterium as a response to the elicitors.

In addition, the involvement of Ca^{2+} on the regulation of bacitracin A production was shown by the addition of ionomycin (positive control) and by the presence of Ca^{+2} channel blocker (verapamil) on cultures of *B. licheniformis*. Although the improved bacitracin A levels were not sustained and disappeared with time, a 15 % increase in the antibiotic level was observed at 32 hrs in cultures treated with ionomycin. This suggests that Ca^{2+} influx plays a crucial role in the bacitracin A overproduction. A comparable finding was made in carrot culture (*Daucus carota* L.) where the addition of calcium ionophore A23187 induced the overproduction of phytoalexins (Kurosaki *et al*., 1987).

The addition of Ca^{2+} channel blocker to the *B. licheniformis* cultures lowered bacitracin A production (in control and test cultures) without affecting the normal growth of the bacterium. The uptake of $CaCl_2$ present in M20 medium, suggested as an important component for the production of bacitracin A (Yousaf, 1997) might be inhibited by the Ca^{2+} channel blocker, and this could be the reason for the low antibiotic production.

These results suggest that Ca^{2+} channels might be directly involved during elicitor-mediated enhancement of bacitracin A production. In plant cells, elicitation has been known to be inhibited by Ca^{2+} channel blockers. The Ca^{2+} channel blockers were found to reduce the enhanced accumulation of anthocyanin in response to sucrose in grape cell cultures (Vitrac *et al*., 2000) and in response to biotic microbial elicitors (Sudha and Ravishankar, 2002). In anion cells, verapamil abolished the elicitor-mediated phytoalexin synthesis (Dmitriev *et al*., 1996)

suggesting that Ca^{2+} might be directly involved in the regulation of phytoalexin synthesis in the culture.

The information obtained in this study records for the first time that, oligosaccharide elicitors are able to induce significant changes in the Ca^{2+} ion flux across the *B. licheniformis* cell membrane. These changes are possibly linked to the effects on the bacitracin A production. Moreover, by the use of a Ca^{2+} channel blocker, this study has shown that Ca^{2+} is essential for bacitracin A production both under non-elicitation and elicitation environments.

4.6.2 Proteomic profile of *B. licheniformis* elicited cultures

All microorganisms respond to biotic and abiotic stress such as nutrient availability, temperature, oxidative stress and the addition of elicitor(s). A better understanding of the physiological and molecular alterations (gene and protein expressions) in response to stress is essential to comprehend the microbial mechanism of stress response. This study unravels changes in the whole proteome and phosphoproteome when oligosaccharide elicitors, OG and MO, are added to *B. licheniformis* cultures.

The results presented in this section are by no means a reflection of the global proteomic changes occurring in this bacterium during the biotic elicitation process. The experiments carried out were designed to selectively capture proteins with a pI between pH 3-10 and/or 4-7, excluding proteins with pI values outside these ranges. Although an effort was made to identify all the differentially expressed protein, ß-mannanase, (enzyme affected by the elicitors - Section 3.3), were not identified from the 2-D gel-based investigations. Three possible reasons could be causing it: 1) this could be because the levels of differential expression of this protein was outside the cut-off employed; or 2) that their expression amounts are below the sensitivity of our system or 3) that it showed significant overlap with other protein species.

Regardless of this limitation, the results discussed in this section indicate significant shifts in the proteome of *B. licheniformis* following the addition of elicitors. As one aim of this work was to identify proteins quantitatively affected

by short and long-term elicitation, it was surprising to observe that although the functional group of the proteins remained the same, the transient elicitor response proteins had less overlap with the later long-term elicitor responses. Furthermore, it was noted that the ratio of up-regulated proteins to down-regulated proteins was larger in short-term response proteins than in long-term response proteins. This may suggest an immediate response of the bacterium to the elicitor, facing a challenging environment, organising its metabolism by up-regulating proteins.

4.6.2.1 Comparative pattern of the whole proteome of *B. licheniformis* subjected to short-term elicitation

Two key regulatory enzymes, belonging to the pentose phosphate and glycolytic pathways were synthesised at elevated levels following the OG and MO short-term elicitation in *B. licheniformis* cultures. Transaldolase (spot 24) is an enzyme involved in the fourth stage of the non-oxidative phase of the pentose phosphate pathway (Stover and Driks, 1999) whereas phosphopyruvate hydratase (Enolase spot 102) is a conserved protein implicated in step nine of the glycolysis pathway (Lehninger, 2002). Although the role of enolase during a stress response is not clear, its up-regulation in response to chilling (Lee *et al*., 2009) and salt stress (Yan *et al*., 2005) in rice roots has been reported. Microbial enolase and transaldolase were also up-regulated in response to metal stress in *Pseudomonas fluorescens* (Sharma *et al*., 2006), cold stress in *Botrytis cinerea* (Pandey *et al*., 2009) and in *Lactobacillus plantarum* in response to lactic acid stress (Pieterse *et al*., 2005), respectively.

In this study we speculated that up-regulation of these energy generating enzymes can be as a result of an increased demand for energy to sustain the amplified metabolic activity caused by the elicitors. Additionally, this energy could be supported by the mannose intake as suggested before. However, further investigation would be required to prove this since proteins directly involved in mannose transport (mannose-PTS - Phosphoenolpyruvate- phosphotransferase systems) and metabolism (phosphomannose isomerase) were not identified in this study. A future experiment may be to monitor changes in mannose transport

proteins in the membrane as these may not vary as much as say the metabolic enzymes.

Seven proteins involved in amino acid biosynthesis (4 up-regulated and 3 down-regulated) were differentially expressed in *B. licheniformis,* after short-term elicitation with OG and MO. Enzyme 3-phosphoshikimate 1-carboxyvinyltransferaseenylalanine (spot 47.3) involved in aromatic amino acids biosynthesis was up-regulated, bifunctional 3-deoxy-7-phosphoheptulonate synthase/chorismate mutase (spot 4), also involved in aromatic amino acid biosynthesis was down-regulated. Although their precise function during elicitation may still be elusive; we speculate that perhaps, the up-regulation of one protein and the down-regulation of another enables *B. licheniformis* to maintain some form of balance during aromatic amino acid synthesis.

Similarly, five spots identified as ketol acid reductoisomerase (2 up-regulated and 3 down-regulated) had also their profile changed in response to OG and MO elicitors after short-term elicitation. Ketol acid reductoisomerase is a key enzyme in the biosynthesis of valine, leucine and isoleucine amino acids (Lehninger, 2002; Veith *et al*., 2004; Mader *et al*., 2004) and therefore changes in its expression could suggest the importance of these amino acids in *B. licheniformis* response to elicitor supplementation. Overall it is worth mentioning that bacitracin A, whose production is so far the main response observed in response to elicitation in *B. licheniformis*, is a complex peptide formed by isoleucine, cysteine, leucine, glutamic acid, phenylalanine (Aftab, 2010), It could be suggested that differences in expression of proteins involved in amino acids biosynthesis might be directly linked to the enhanced bacitracin biosynthesis. However, more work is required since proteins directly involved in bacitracin biosynthesis (bacitracin synthetase ABC) were not identified in this study, as responding to the presence of elicitors.

Two spots identified as oxalate decarboxylase were differently expressed (one up-regulated and on down-regulated) as a response to short-treatment of OG and MO in *B.licheniformis*. In filamentous fungi, oxalate decarboxylase is induced in the presence of oxalic acid (a metabolic waste compound synthesised through the

linkage of the TCA and glyoxylate cycles) (Dutton and Evans, 1996; Just *et al*., 2007; Makela *et al*., 2010). Fungi also produce oxalate in order to stimulate the activity of an extracellular Mn peroxidase involved in lignin degradation; in this context, oxalate decarboxylase has been reported to confer protection against excess oxalic acid in fungi (Dunwell *et al*., 2000).

However, in *B. subtillis*, the absence of induction of the oxalate decarboxylase enzyme by oxalic acid suggests that this enzyme has a role that is different to that in fungi (Tanner and Bornemann, 2000). Costa *et al*., (2004) and Antelmann *et al*., (2007) showed that in *B. subtillis*, oxalate decarboxylase is a spore-associated protein. Though the specific role of oxalate decarboxylase within the spore coat is unclear, Nicholson, (2002) has suggested that oxalate decarboxylase might only play a structural role in coat assembly, or it could also function in spore protection, during germination. It is plausible to suggest that, perhaps OG and MO affect *B. licheniformis* sporulation which in turn would require the expression of this spore-associated protein. Nevertheless, further investigation is required in order to prove this association.

The results also suggest that OG and MO might be acting as stress factors, since proteins involved in a general stress response (NAD synthetase and YceD) and antioxidant enzymes known to maintain ROS levels (superoxide dismutase and alkyl hydroperoxide reductase), were also differentially expressed in *B. licheniformis* after short term-elicitation. In *Bacillus spp.* NAD synthetase is a member of a σ^{B}-dependent general stress regulon (Rey *et al*., 2004; Veiga-Malta *et al*., 2004). NAD synthetase catalyses the synthesis of NAD from either NH_3 or glutamine and nicotinic acid adenine dinucleotide (Antelman and Hecker, 1997). Although its role in stress adaptation is not clear, transcriptional studies have shown that NAD synthethase is strongly induced (in a sigma σ^{B}-dependent manner) in response to heat, ethanol, salt stress or after glucose starvation (Hecker *et al*., 1996; Antelman *et al*., 1997). Likewise, the putative stress response protein (YceD), also up-regulated, has been reported to be involved in the maintenance of cell integrity (Cao *et al*., 2002), during alkaline (Huang *et al*., 1999) and salt stress (Höper *et al*., 2005). Interestingly, YceD is also a family member of a σ^{B}-dependent general stress regulon (Höper *et al*., 2005). The σ^{B}-dependent general

stress regulon controls the expression of more than 100 stress genes related to heat, acid, ethanol, salt stress and starvation (Volker *et al*., 1999). MO and OG may generate a stress condition which in turn induces the expression of the σ^{B}-dependent general stress proteins.

Antioxidant enzymes involved in the regulation of ROS were also differentially expressed. Thioredoxin known to play a crucial role in maintaining cellular redox homeostasis (Karlenius and Tonissen, 2010) was up-regulated in response to short-term elicitation. In *B. subtillis*, thioredoxin has been showed to defend cells against oxidative stress by maintaining the reducing environment in the cell cytoplasm (Zhang *et al*., 2006). In addition, two species of superoxide dismutase, and an alkyl hydroperoxide reductase were down-regulated. However, interestingly, the phosphorylation of these proteins and the overall activity of superoxide dismutase was increased (section 3.6.1.2). Therefore we suggest that the activity regulation of these enzymes might occur via PTM and not through their expression. A similar pattern was observed in the expression of a translocation dependent antimicrobial spore component, which will be further discussed in the next section.

4.6.2.2 Effect of the elicitors on the phosphoproteome of *B. licheniformis* subjected to short-term elicitation

Serine, threonine and tyrosine protein phosphorylation is the most extensively studied post-translational modification in bacteria, where it plays an important role in signal transduction and cellular regulation (Misra *et al*., 2011). Changes in the phosphorylation state of proteins under abiotic stress (Lévine *et al*., 2006; Eymann *et al*., 2007) and the whole phosphoproteome (Maced *et al*., 2007) in *Bacillus* spp. have been reported. In this study, for the first time, we discuss changes in the phosphoproteome of *B. licheniformis* in response to the oligosaccharide elicitors OG and MO.

Multiple enzymes within cells produce reactive oxygen species (ROS). In eukaryotic cells, the production of ROS occurs by oxidative phosphorylation through the mitochondrial respiratory chain. Cells use ROS as stress signalling entities and as antimicrobial agents (Finkel and Holbrook, 2000; Archambaud *et*

al., 2006). However, 'over-production' of ROS is harmful to cells and can damage nucleic acids, proteins and lipids (Imlay, 2003). Hence the cell maintains an oxidative defence system that includes both non-enzymatic (e.g. glutathione and thioredoxin) and enzymatic (e.g. superoxide dismutase (SOD), alkyl hydroperoxide reductase (Ahp) and catalase detoxification) mechanisms which remove ROS and restore the redox balance (Mager *et al*., 2000). SOD catalyse the conversion of the superoxide radical anion to H_2O_2 and catalase and Ahp serves to detoxify H_2O_2. The expression of both SOD and Ahp enzymes are reported to be tightly controlled at the transcriptional and post-transcriptional levels (Lee *et al*., 1999; Archambaud *et al*., 2006). Archmbaud *et al*., (2006) reported the regulation of SOD by serine/threonine phosphorylation in cultures of *Listeria monocytogenes*. Lee *et al*., (1999) demonstrated that Ahp is highly modulated by phosphorylation in *Saccharomyces cerevisiae* as a response to oxidative stress.

Previous elicitation studies, (Murphy, 2008), demonstrated that when multiple biotic elicitors, OG and MO, are added to *B. licheniformis* cultures a reduction in intracellular ROS levels occurs concomitantly with an increase in catalase activity. Sangworachat, (2006), showed the same pattern in the filamentous bacteria (*Streptomyces spp.*). It was suggested that the reduction of ROS levels observed upon elicitor addition could be due to the enhanced production of antioxidant enzymes. Our study has demonstrated that the addition of the oligosaccharides caused a 2.1 fold increase in the phosphorylation of these two protective enzymes of oxidative stress, the antioxidants, SOD and Ahp, in the elicited state as compared to control cultures (Figure 3.36 and Table 3.6). In *B. subtillis* the enhanced activity of SOD and Ahp is known to occur in response to oxidative stress (Dowds, 1994).

In this context, the increased phosphorylation state of gentisate 1, 2 dioxygenase (spot 93), could be linked to the physiological requirement of the *B. licheniformis* cells to recycle any damaged proteins caused by ROS. Gentisate 1, 2 dioxygenase is a key intermediate enzyme involved in the biodegradation of a large number of simple and complex aromatic compounds produced by microorganisms (Kiemer *et al*., 1996; Zhao *et al*., 2004). This enzyme may also be involved in the

degradation of non-required proteins and dormant enzymes, a measure required under stress to regulate the "biochemical economics" of the cell. Changes in the phosphorylation state of a spore associated protein; oxalate decarboxylase (Dashek and Micoles, 1997) (discussed in section 4.5.2) and the translocation dependent antimicrobial spore component, were also observed in response to OG and MO. In *B. subtillis*, the translocation dependent antimicrobial spore component (spots 59 and 2.2) has been reported to be governed by a multicomponent phosphorelay and to be affected by stress (Tam *et al*., 2006). It exhibits antibacterial activity against a variety of Gram-positive and Gram-negative bacteria, thus providing sporulating cells with a competitive advantage during the early stages of development as well as providing protection during and after germination (Stover and Driks, 1999). Many microorganisms are naturally programmed to produce spores when the specific growth rate decreases below a certain level as a result of nutrient deficiency or as a response to stress stimulation (Jarvis and Johnson, 1947).

Elicitation studies in *Penicillium* spp. and *Streptomyces* spp. have shown that addition of elicitors to cultures enhances the production of antibiotics and initiates earlier sporulation compared to control cultures (Radman, 2002; Sangworachat, 2006). In *Bacillus* cultures, antibiotic production is directly associated with sporulation (Shaeffer, 1969). According to the author, antibiotics such as bacitracin A act as differentiation effectors which are essential for the formation of spore and proper timing of spore germination. This association is partly based on the observation that inhibitors of spore formation in stationary phase often inhibit the production of antibiotics (Bernlohr and Novelli, 1959). It could be suggested that the increased activity of these proteins might be a consequence of the overproduction of bacitracin A in response to the elicitors' supplementation.

Changes in the phosphorylation state of enzymes involved in energy generation, phosphopyruvate hydratase (enolase) (spot 102, Tavichakorntrakool *et al*., 2009), transaldolase (spot 24) involved in the pentose phosphate pathway (Stover and Driks, 1999) and NAD synthetase, enzyme involved in NAD^+ synthesis (Lima *et al*., 1992) were also observed. In *B. subtillis*, it is well established, that the activity of these enzymes is controlled by phosphorylation of serine/threonine/tyrosine

residues (Macek *et al*., 2007). These enzymes were also shown to have their total expression increased by the elicitors (section 3.6.1 and 3.6.2). The fact that the expression and activity of these enzymes were both enhanced during short-treatment with OG and MO, gives additional support to the suggestion that these enzymes might play a key role in the adaptation process of *B. licheniformis* during elicitation.

The phosphorylation state of proteins involved in a wide range of amino acid biosynthetic steps such as ketol acid reductoisomerase (involved in valine, leucine and isoleucine biosynthesis (Irmler *et al*., 2006) whose function was discussed in section 4.6.2.1), and phosphoserine aminotransferase (PSAT) increased under elicited conditions. PSAT is thought to be involved in the phosphorylated L-serine biosynthesis pathway (Ali and Nozaki, 2006). L-Serine serves as a building block for protein synthesis and plays an important role in various metabolic pathways for the generation of essential compounds, such as glycine, cysteine and purines (Snell, 1984; Snyder and Kim, 2000). Unlike ketol acid reductoisomerase, where both its expression and activity (via phosphorylation) were affected, only changes in PSAT phosphorylation were seen in this study. Although these proteins are not directly involved in the overproduction of bacitracin A, changes in their expression and phosphorylation state, may affect amino acid biosynthesis during the elicitation process. Figure 4.3 shows the function of the proteins involved in energy generation and amino acid biosynthesis, affected by the short-term elicitation in *B. licheniformis* culture.

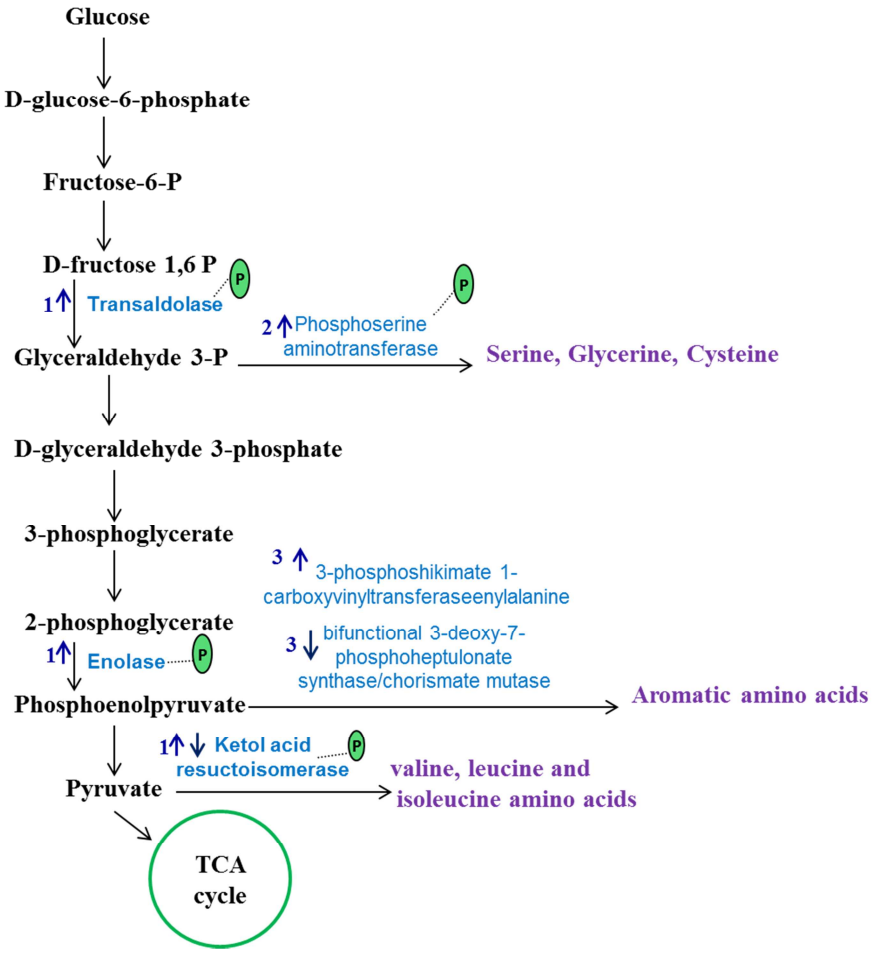


Figure 4.3 The central carbohydrate metabolic network of *B. licheniformis* showing the role of the proteins affected by short-term elicitation. In black are the 6 precursor metabolites. Enzymes in blue: (1) Protein affected at levels of expression and phosphorylation; (2) Protein with phosphorylation state affected; (3) Protein with expression levels affected by OG and MO during short-term elicitation. In purple contain the downstream end-products of the biosynthetic pathways that branch out from central carbon metabolism. Arrows: ↑ (up-regulated) and ↓ (down-regulated). (P) (Green) phosphoryl group added to the protein (Adapted from Lehninger, 2002).

Even though this phosphoproteome study revealed significant changes occurring in the phosphorylation state of proteins as a response to OG and MO addition, none of them were directly linked with mannose uptake and metabolism or bacitracin A biosynthesis. This is presumptively due to the use of Pro-Q Diamond which only detects proteins where the phosphoryl group has been added at their serine/tyrosine/threonine residues. The regulation of the enzymes involved in the mannose and bacitracin processes are reported to occur via histidine/aspartate phosphorylation which is often coupled with a two component system.

According to Fabret *et al*., (1999) and Stock *et al*., (2000), two-component systems function as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental inputs such as nutrient availability, pH, salinity, temperature and the presence of other microorganisms. The typical system consists of a histidine protein kinase sensor (HK), containing a conserved kinase core, and a response regulator protein (RR), containing a conserved regulatory domain. Extracellular stimuli are sensed by, and serve to modulate the activities of the HK. The HK transfers a phosphoryl group to the RR, in a reaction catalysed by the RR. Phosphotransfer to the RR results in activation of a downstream effector domain that elicits a specific response.

Histidine phosphorylation is prominent in bacterial carbohydrate transport and metabolism (phosphoenolpyruvate: carbohydrate phosphotransferase transport system - (PTS). Bacteria rely on this system to sense, import and regulate metabolic activities related to carbohydrate degradation (enzymatically) and consumption (Postma *et al*., 1993). In *Bacillus* spp. the presence of the PTS and its role in sensing carbon source inputs (Kornberg and Miller, 1972; Tangney *et al*., 1993; 1996) including, mannan oligosaccharide (Reizer *et al*., 1999) and mannose (Barabote and Saier, 2005; Sun and Altenbuchner, 2010) are known. Although there are no reports showing the relationship between stress response and bacitracin A production, we speculate that perhaps via a two-component system, *B. licheniformis* senses MO/mannobiose as a hostile factor and responds to it by overproducing bacitracin A. This speculation is supported by the fact that mannan oligosaccharides are a component of fungal cell walls such as *Candida* spp. and *Saccharomyces cerevisiae* (Domer, 1989; Huang *et al*., 2010). In plant systems, the addition of oligosaccharides to the culture is well known to trigger a defence response mechanism which results in production or overproduction of plant secondary metabolites (Shibuya and Minami, 2001).

4.6.2.3 Reactive oxygen species, SOD and catalase activity

An investigation of the ROS, SOD and catalase activity in elicited and control cultures was carried out after the observed alterations in the phosphorylation state of proteins expressed under elicitation conditions (section 3.6.1.2) and in light of previous studies suggesting elevated redox enzyme activity in *B. licheniformis* (Murphy, 2008) and *Streptomyces* spp. (Sangworachat, 2006).

Aerobic organisms continually metabolise oxygen through oxidative systems, such as the electron respiratory transport chain (Soballe and Poole, 1999; Brooijmans, 2008). Occasionally, oxygen undergoes incomplete one-electron reduction to form highly reactive molecules commonly called reactive oxygen species (ROS) (Karlenius and Tonissen, 2010). Within the cells, ROS are constantly formed under normal physiological conditions and it has been reported that they act as signalling molecules and redox regulators. Nevertheless, their overproduction (oxidative stress) can cause significant damage within cells (Kang *et al*., 2002). Hence, the cell maintains an oxidative defence system that includes both non-enzymatic (e.g. glutathione) and enzymatic (e.g. SOD, Ahp and catalase) detoxification mechanisms which destroy ROS and restore the redox balance (Mager *et al*., 2000). SOD catalyses the conversion of the superoxide radical anion to H_2O_2. Catalase and Ahp serve to detoxify the H_2O_2.

In this study, the effect of oligosaccharide elicitors OG and MO on ROS, SOD and catalase levels in *B. licheniformis* cultures were investigated during the first 30 min post-elicitor addition at 24 hrs. The results showed that increased ROS levels observed during the course of the experiment were concomitant with enhanced SOD activity. However, catalase activity was reduced by short-term elicitation (Figure 3.43). This suggests that at least during short-term elicitation, as showed by the phosphorylation study, *B. licheniformis* uses the Ahp enzyme instead of catalase to scavenge H_2O_2 generated by SOD. Ahp has been reported to be a more efficient scavenger of trace H_2O_2 than catalase (Seaver and Imlay, 2001).

Earlier elicitation reports, using longer sampling points, showed higher activity of catalase concomitant with a reduction in intracellular ROS levels in cultures of

B. licheniformis and *Streptomyces* spp. treated with the same elicitors (Sangworachat, 2006; Murphy, 2008). This implies that under short-term elicitation, ROS levels are immediately affected by OG and MO, and then cells respond by activating the antioxidant enzymes SOD and Ahp, whereas during long term elicitation, ROS levels are kept stable possibly by the expression of SOD and catalase as shown before.

Another possible reason for the increased generation of ROS might be related to the changes in the metabolic rate caused by the mannose consumption. Barbaneagra *et al*., (2011), reported changes in ROS and antioxidant enzyme activity associated with carbon source uptake in *Rhizopus nigricans* cultures.

4.6.3 Comparative pattern to the whole proteome of *B. licheniformis* subjected to long-term elicitation

A comparative proteome analysis of *B. licheniformis*, responding to long-term exposure to OG and MO, showed that spore-associated proteins were abundantly affected by the elicitors. Five different spots (Table 3.10) identified as oxalate decarboxylase (Antelmann *et al*., 1997) and three different spots identified as translocation dependent antimicrobial spore component (Stover and Driks, 1999), (which function as spore associated proteins were already covered in section 4.6.2), were differentially expressed during long-term elicitation. As mentioned before, antibiotic production is directly associated with sporulation in *Bacillus* spp. (Schaeffer, 1969), where inhibition of spore formation during stationary phase often inhibits antibiotic production (Bernlohr and Novelli, 1959). An earlier study measuring the effect of elicitors on OG and MO on bacitracin A production (Murphy, 2008), showed that the highest increase in the antibiotic production (as compared to the control culture) was obtained 48 hrs after inoculation. Samples taken from *B. licheniformis* culture, to investigate changes in the proteome occurring during the same long-term exposure to OG and MO, were at 48 hrs of growth.

Two key regulatory enzymes, involved in glycerol and ethanol metabolism, were down-regulated in *B. licheniformis* in response to long-term elicitation. As by-products of its own metabolism, *B. licheniformis* cells are able to recycle glycerol

and ethanol. It utilises them as energy sources, during starvation periods, via glycolysis and the TCA cycle (Figure 4.4) with the help of glycerol dehydrogenase (Zhao *et al*., 2009; Ruzheinikov *et al*., 2001) and aldehyde dehydrogenase (Picchiottino and Lee, 2002), respectively. Nevertheless, the suppression of these proteins by long-term elicitation supports the indication of the presence of an additional energy source in the medium. As previously shown, L-glutamic acid, the only carbon source present in M20 medium, is depleted by the early stationary phase, therefore, we suggest that this extra energy may be attained, at least to some extent, by mannose uptake (as an MO degradation product).

Down-regulation of threonine synthetase (Schildkraut and Greer, 1973) and ketol acid reductoisomerase, (Irmler *et al*., 2006) involved in threonine and in valine, leucine and isoleucine biosynthesis, respectively, suggest that at this stage the biosynthesis of these amino acids is not required. Conversely, a key enzyme of the TCA cycle was up-regulated. Isocitrate dehydrogenase (spots 239 and 250) converts isocitrate to α-ketoglutarate while reducing NAD^+ to NADH (Singh *et al*., 2002) (Figure 4.4). This process ensures continuation of the cycle, but more importantly serves as a source of glutamate, purines, arginine, proline and glutamine (Lehninger, 2002), implying that these amino acids might play an important role in the *B. licheniformis* adaptation to long-term elicitation by OG and MO. In section 3.5 we demonstrated that elicitors caused changes in calcium flux in *B. licheniformis*. Calcium has been reported to enhance cellular metabolism by activating the phosphorylation of three enzymes (pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase) of the TCA cycle (Brookes *et al*., 2004). This might explain enhanced expression of this enzyme and consequently, TCA cycle flux and amino acid biosynthesis.

In bacterial translation, three elongation factors, EF-Tu, EF-Ts and EF-G are responsible for escorting aminoacyl tRNAs to the ribosome and for the translocation of the ribosome along the mRNA (Cooper, 2000). A 1.7 fold increase in the expression of EF-Tu and EF-G was observed in *B. licheniformis* cultures long-term supplemented with OG and MO. An increase in EF-Tu and EF-G during acid stress has been reported in *Streptococcus mutants* (Len *et al*., 2004)

and in *E. coli* where these elongation factors were shown to behave like a chaperones towards unfolded and denatured proteins (Kudlicki *et al*., 1997; Caldas *et al*., 1998 and 2000). EF-Tu, for example, recognises the same hydrophobic binding motifs in proteins as the chaperone DnaK (Malki *et al*., 2002). Furthermore, EF-Ts acts as a folding template in a chaperone-like manner towards its substrate protein, EF-Tu (Krab *et al*., 2001). Although its function is not clear, in a proteomic analysis of the alkaliphilic *Bacillus sp.* N16 showed up-regulation of these elongation factors in response to growth in different carbon sources, including mannose (Li *et al*., 2011). Figure 4.4 shows the function of the proteins involved in energy generation and amino acid biosynthesis, affected by the long-term elicitation in *B. licheniformis* culture.

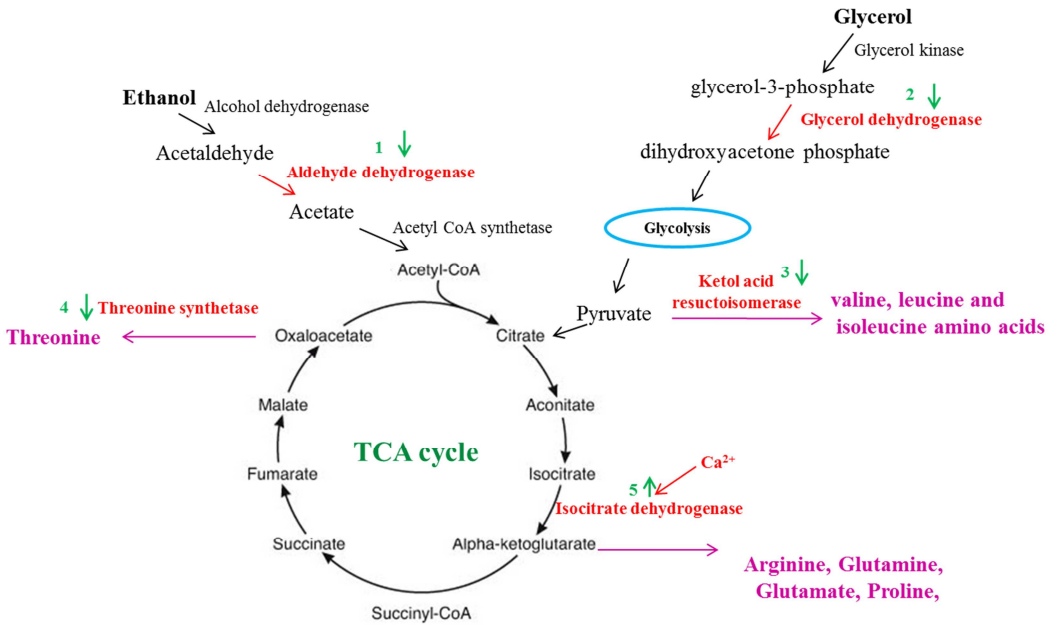


Figure 4.4 The central carbohydrate metabolic network of *B. licheniformis* affected by long-term elicitation. Arrows (green): ↑ (up-regulated) and ↓ (down-regulated) and numbers (green) are enzymes: (1) enzyme involved in ethanol metabolism; (2) enzyme involved in glycerol metabolism; (3) enzyme involved in valine, leucine and isoleucine biosynthesis, (4) enzyme involved in threonine biosynthesis and (5) key enzyme of TCA cycle. (Adapted from Lehninger, 2002).

4.7 Elicitation mechanism in *B. licheniformis* cultures

A hypothetical mechanism of elicitation in *B. licheniformis* has been proposed (Murphy, 2008) and it was used as starting point for the further investigation carried out in this thesis.

Based on the results obtained, we suggest that, bacitracin A overproduction, is a consequence of the cumulative events occurring during the initial interaction of *B. licheniformis* cells, under a specific physiological state of growth, with the oligosaccharide elicitors (OG and MO) (Figure 4.5). Elicitation in *B. licheniformis* is believed to be initiated through the recognition of elicitors by an oligosaccharide-receptor/sensor presumed to be present on the bacterium cell membrane. As a result of the elicitor recognition by the sensor, a wide range of biological responses, including: changes in Ca^{2+} influx (section 3.5), changes in expression and phosphorylation state of proteins (section 3.6), changes in ROS levels (section 3.6.3.1); catalase and SOD activity (section 3.6.3.2); production of the enzymes capable of degrading MO (section 3.2.3) and overproduction of bacitracin A (section 3.4), were observed in *B. licheniformis* cultures (Figure 4.5).

We have shown that *B. licheniformis* is capable of degrading MO by the enzyme ß-mannanase. The individual addition of MO degradation products (mannose, mannobiose and mannohexaose) to cultures of the bacterium indicated that in fact mannobiose is the entity which has the biological functionality present as part of MO, since its addition to the bacterium culture improved bacitracin A production to the same extent as MO. It was also observed that the degradation products of MO are further degraded by the mannanase enzyme to the end product, mannose. Since *B. licheniformis* is known to be capable of utilising mannose as a carbon source (Durand *et al*., 1979; Nilegaonkar *et al*., 1996; Van Dyk, 2009) and for production of bacitracin A (Qadeer *et al*., 1988), we theorise that mannose is transported into the cells, incorporated into the carbohydrate metabolic pathways, and the extra energy generated by this process is then used to support the increased metabolic requirements of the elicitation process. Extra energy would be required for amino acid biosynthesis and for the enhanced production of metabolites such as bacitracin A. This speculation can be supported by the fact

that the majority of proteins affected by elicitors, with altered expression and phosphorylation state, were related to carbohydrate metabolism/energy generation and amino acid biosynthesis (Section 4.5.2).

Protein kinases are signal transducing enzymes that in *Bacillus* spp. work together with response regulators (the target protein) (Wollanin and Stock, 2002). Bacterial histidine, serine/threonine and tyrosine kinases (Manu-Boateng, 2007) activate numerous enzymes through phosphorylation (Wollanin and Stock, 2002). The phosphorylation state of proteins affected by the elicitors were mostly those involved in energy generation and stress responses (section 4.5.2), suggesting that the presence of elicitors creates a stress scenario where scavenging of ROS is subject to regulation mainly via phosphorylation. Additionally, an increase in protein phosphorylation, calcium transients and ROS, could activate transcriptional factors involved in the enhanced transcription of genes encoding for proteins implicated in the biosynthesis and transport of bacitracin A, as earlier reported in *B. licheniformis* cultures treated with elicitors (Murphy, 2008).

Changes in Ca^{2+} influx in *B.licheniformis* cultures have been shown to occur in response to elicitors (section 3.5). Changes in Ca^{2+} influx activate a number of cellular responses. For instance, Ca^{2+} has been reported to enhance cell metabolism by activating the phosphorylation of 3 enzymes (pyruvate dehydrogenase, isocitrate dehydrogenase and α- ketoglutarate dehydrogenase) of the TCA cycle (Brookes *et al*., 2004).

During the proteome studies isocitrate dehydrogenase was up-regulated in response to elicitors (section 4.5.2), confirming the existence of a high metabolic activity in cultures treated with elicitors.

High respiration activities caused by the extra energy may mean that more electrons would leak from the respiratory chain and ROS levels would be expected to be higher than during normal respiration (Brookes *et al*., 2004; Yan *et al*., 2006c). An increase in ROS levels were observed in MO and OG supplemented cultures. ROS can also play an important part in signalling, as they are able to activate transcription factors (Thannickal and Fanburg, 2000). In response to the high levels of ROS, cells respond by activating a set of antioxidant

enzymes responsible for ROS regulation. Increased phosphorylation of Ahp and SOD activities were observed in elicited cultures compared to control cultures (section 4.5.3).

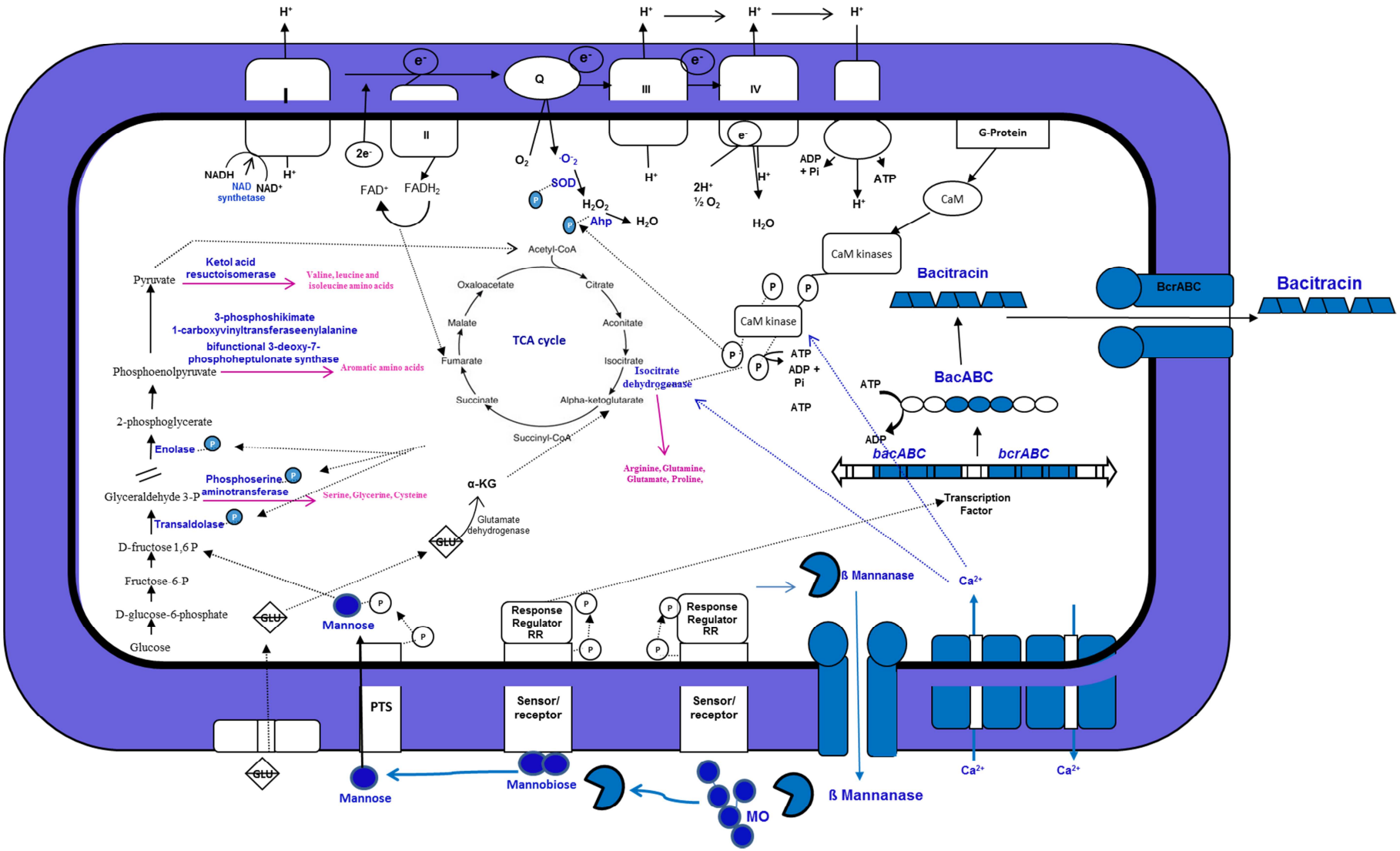


Figure 4.5 Early events observed during MO elicitation in *B. licheniformis*. Accomplishments done marked as blue.

Chapter 5

5.0 Conclusion

5.0 Conclusion

This work has brought together information on the mechanism by which enhanced bacitracin A production is achieved in *Bacillus licheniformis* cultures through the addition of biotic oligosaccharide elicitors. A range of metabolic responses were observed and a mechanism was suggested for elicitation in *B. licheniformis* cultures.

This study, for the first time, showed that minimal and maximal effective concentrations for OG, OM and MO elicitors exist in *B. licheniformis* culture. This suggests that the effects generated by these elicitors in this bacterium are dose-dependent. Continuous stimulation of *B. licheniformis* by the MO elicitor in chemostat culture did not enhanced bacitracin A production. It is presumed that a specific physiological state of growth needs to be reached before the *B. licheniformis* cells can perceive the MO, receive stimulation and generate a response. This novel information reveals that the overproduction of bacitracin A is not only based on the MO addition to the culture of *B. licheniformis*, but it is the outcome of a combined interaction of MO and the changes in cell physiology during the growth of the bacterium.

Novel data has been obtained demonstrating that, at least in *B. licheniformis*, mannobiose, as an enzymatic degradation product of MO, is the entity responsible for the biological functions observed in MO. This study also showed that following the stimulation of the cell; mannobiose is further degraded into mannose by the ß-mannanase, which in turn disappears with time. As *B. licheniformis* is capable of utilising mannose as carbon source, it is suggested that mannose is incorporated into carbohydrate metabolic pathway. The extra energy would be used to support the increased metabolic events taking place during elicitation process. This speculation is supported by the fact that the majority of proteins (expression and phosphorylation stated) affected by the elicitors were related to the carbohydrate metabolism/energy generation and amino acid biosynthesis. The *B. licheniformis* proteome profile also suggest that OG and MO might be acting as stress factors, since changes in phosphorylation and activity of proteins known to maintain ROS levels were also observed.

This study also denote for the first time that oligosaccharide elicitors are able to induce significant changes in the intracellular Ca^{2+} ion flux across the *B. licheniformis* cell membrane, and these changes are possibly involved in the effects caused on the bacitracin A production.

The novel findings of this project, together with previous reports, contribute in the unravelling of the overall mechanism of elicitation in *B. licheniformis* cultures. A better understanding of the mechanism of action of elicitors in bacterial cells, would, potentially, have significant impact on the industrial production of commercially useful microbial by-products.

Chapter 6

6.0 Future Work

6.1 Oligosaccharide receptor / sensor

It has been suggested that the elicitation process might be initiated at cell surface, through the perception of the elicitor by a specific "receptor / sensor" (Radman, 2002; Murphy, 2008). However, no elicitor binding receptor / sensor has been identified so far. Theoretically, these "receptor / sensor (s)" are likely to be lectins or PTS (phosphoenolpyruvate: sugar phosphotransferase system) type, respectively. Therefore:

a. Lectin type oligosaccharide-receptor studies in *B. licheniformis* could provide information regarding the existence of specific oligosaccharides-receptor in the cells surface of these cultures and the nature of interaction between the bacterial lectins and the different elicitors. Carbohydrate microarrays could serve as a useful tool for rapid screening of binding profiles of different elicitors to a particular culture, as well as the characterisation of carbohydrate cell interaction events. An important advantage of this method is the use of intact cells for screening (Paz and Seeberger, 2006).

The oligosaccharide-receptor studies could also be carried out by labelling the oligosaccharide elicitors. This method could trace the path of the oligosaccharide and would provide information on the involvement of oligosaccharide-specific receptor(s) and their binding capacity towards the different oligosaccharide-based elicitors. Extraction and identification of the receptor(s) could be done by using Western Blot technique or the use of affinity chromatography based on the specific biological interaction between lectin receptor(s) and the ligands (oligosaccharides elicitors).

b. In this thesis we showed that mannose is the end product of the gradual degradation of MO by ß-mannanase. As mannose disappeared from the liquid culture as the fermentation proceeds, we speculate that mannose was consumed by *B. licheniformis*. In *Bacillus* species sugars are normally transported and phosphorylated by the phosphoenolpyruvate: sugar phosphotransferase system (PTS) (Saier, 1977). The investigation of the transport of mannose and its non-metabolisable analogue in a mutant strain

lacking the phosphor-carrier proteins of the PTS system, would provide further information regarding potential role of PTS and the metabolised mannose during the elicitation process.

c. This thesis also showed that mannobiose is the entity which has the biological function observed in MO. It has been reported that PTS regulation network not only controls carbohydrate uptake and metabolism but also acts as a sensory system. In this context, PTS system is also able to convert external stimuli into intracellular signal (Kremling *et al*., 2007). The occurrence of protein, capable to sense and respond to the presence of mannobiose, phosphotransferase system enzyme II (PSEII), (Kunst *et al*., 1997) in *Bacillus* spp. is known (Rey *et al*., 2004). An investigation involving a mutant strain defective in PSEII (Harwood *et al*., 1976) would provide further information regarding the mannobiose role on the signalling events observed in *B. licheniformis* which results in the overproduction of bacitracin A.

6.2 Fate of elicitors in *B. licheniformis* cultures

a. In this study, it was observed that the precise detection of MO in liquid culture of *B. licheniformis* by phenol sulphuric acid assay was masked due to the production of exopolysaccharides by the bacterium. For this reason a specific method for MO detection in liquid culture using HPAEC and TLC chromatography was used. However, the fate of OG and OM in *B. licheniformis* still needs to be revealed. For this reason, a specific protocol for HPAEC and TLC chromatography must be employed to investigate the fate of these elicitors for the whole duration of the fermentation. To do this, oligosaccharide-specific HPAEC column such as Carbo Pac 200 could be used.

6.3 Investigation of enzyme activity capable of degrading OG and OM

This study showed that addition of MO induced ß-mannanase enzyme, which degrades MO in mannose residues. The possible degradation of OG and OM by the *B. licheniformis* could be revealed by measuring enzyme activity of

alginate lyase group, guluronate lyase and mannuronate lyase; both enzymes are known to be responsible for catalysing the degradation of OG and OM, respectively (Heyraud *et al.*, 1998; Wong *et al.*, 2000). In addition, studies have demonstrated that *Bacillus* sp. is capable to synthesise these enzymes (Wong *et al.*, 2000).

6.4 Sporulation studies

Elicitation studies in *Penicillium* sp. and *Streptomyces* sp. have shown that addition of elicitors to the cultures enhances the production of antibiotics and initiates earlier sporulation compared to the control cultures (Radman, 2002; Sangworachat, 2006). Many polypeptide antibiotics produced by *Bacillus* cultures have been reported to affect the spore formation directly or indirectly (Demain and Piret, 1978). Furthermore, the proteome results in this thesis revealed that spore-associated proteins were up-regulated in response to OG and MO. Therefore studying the effect of elicitors on sporulation and its relation to bacitracin A production might provide useful information regards the possible regulatory functions of the elicitor.

6.5 Investigation of the effect of elicitor addition on the phosphorylation of cytosolic proteins

In this study serine, tyrosine and threonine phosphoproteome revealed significant changes in the activity of proteins in response of OG and MO. However, none of them were directly linked to mannose uptake and bacitracin A biosynthesis. The lack of evidence could be due to the method used in this study for the detection of phosphoproteins. Pro-Q Diamond only detects proteins which the phosphoryl group adds at their serine/tyrosine/threonine residues. Therefore, investigation of the proteins phosphorylated at histidine/aspartame residues would suggest the signal transduction mechanism leading to elicitation in *B. licheniformis*. This could be done as described by Besant and Attwoodn, (2009) or by Lapek *et al.*, (2010).

6.6 Effect of elicitation on antioxidant enzymes in *B. licheniformis*

In this study changes in ROS levels and activity of enzymes SOD and catalase were successfully determined. As changes were also observed in phosphorylation of the ROS scavenger, thioredoxin and alkyl hydroperoxide reductase (Ahp), it would be useful to investigate the activity of these enzymes in control and elicited cultures. The presence of thioredoxin and Ahp enzymes has been reported in *Bacillus* cultures. Thioredoxin and Ahp activity could be determined by the methods described by Bartolucci *et al*., (1997) and Seaver and Imlay, (2001), respectively.

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